Molecular characterization of a fungal aldehyde dehydrogenase in the *Tricholoma vaccinum*-spruce ectomycorrhiza

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ABBREVIATIONS

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
APS	Adenosine 5´ phosphosulphate
BAld	benzaldehyde
cDNA	complementary DNA
CTAB	cetyltrimethylammonium bromide
DAPI	4,6-Diamidino-2-phenylindol
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
gpd	glyceraldehyde-3-phosphate dehydrogenase
IAA	indole-3-acetic acid
IAAld	indole-3-acetaldehyde
IPTG	isopropyl β-D-1-thiogalactopyranoside
ITS	internal transcribed spacer
LB	luria bertani
MMN	Modified Melin-Nokrans
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PIPES	1,4-Piperazinediethanesulfonic acid
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SOC	super optimal broth with catabolite repressor
SSC	sodium chloride sodium citrate
TAE	tris-acetate-EDTA
TAIL-PCR	thermal asymmetric interlaced-PCR
TIBA	2, 3, 5 – triiodobenzoic acid
Tris	tris-(hydroxymethyl)-aminomethane
VA	vesicular-arbuscular
vol.	volume
X-Gal	5-Bromo-4-chloro-3-indoxyl-β-D-galactoside

SUMMARY

Understanding of ectomycorrhiza functioning has been slowed down by less investigations of this symbiotic association at the molecular level. In this study, the possible role of a specific fungal aldehyde dehydrogenase (ALDH) in the host-specific mutual symbiosis between the basidiomycete fungus *Tricholoma vaccinum* and its compatible host plant spruce (*Picea abies*) was investigated. Also, the involvement of indole-3-acetic acid (IAA) in ectomycorrhiza formation, which has been controversially observed and discussed in literature, was investigated in detail.

As a first step, the ectomycorrhiza-specifically expressed gene *ald1* was isolated from *T. vaccinum*. Sequence analysis showed that the ORF of *ald1* is interrupted by 16 introns. The conceptually translated protein, Ald1, of 502 amino acids with a predicted molecular mass of 53 kDa was subsequently confirmed by Western blotting. An alignment of Ald1 with other 53 specific fungal ALDHs, representing all major phyla in the kingdom of fungi, was used to re-investigate the evolutionary relationships in this enzyme family. The phylogenetic reconstruction, under Bayesian inference, revealed that, with the exception of chytridiomycota, fungal ALDHs, which clustered in distinct taxonomic groups in the phylogram, underwent two major duplication events during evolution resulting in multiple ALDH paralogs, with specifically high number of paralogs in higher fungi.

Stress Response Elements (STREs) were observed in the promoter region of *ald1*, suggesting a possible role of stress induction for this gene. This prompted us to investigate the possible aldehyde- and alcohol-mediated stress induction of *ald1* expression by real time RT-PCR, which revealed significantly increased gene expression upon addition of 0.1 mM indole-3-acetaldehyde (IAAld), 0.1 mM benzaldehyde or 0.01% ethanol. Furthermore, heterologous expression of *ald1* in *Escherichia coli* and subsequent *in vitro* enzyme activity assay demonstrated the oxidation of various aldehydes with different kinetics using both NAD^+ and $NADP^+$ as cofactors.

In order to understand the biological function of this gene in *T. vaccinum*, it was overexpressed in the fungus using *Agrobacterium tumefaciens*-mediated transformation (ATMT). Functional analysis showed that Ald1-overproducing transformants significantly reduced ethanol stress. These results unequivocally demonstrated the ability of Ald1 to circumvent ethanol stress, a critical function in ectomycorrhizal habitats. In addition, the induction of *ald1* expression by IAAld suggests that the gene might be involved, at least partly, in production of indole-3-acetic acid (IAA).

Investigation of IAA biosynthesis in *T. vaccinum* showed that the fungus produces detectable levels of IAA only in the presence of its precursors, presumably through a tryptophan-indole pyruvate-IAAld-IAA biosynthetic pathway. Furthermore, this phytohormone was, for the first time in ectomycorrhizal fungi, found to increase growth and hyphal ramification of *T. vaccinum*. Taken together, these results strongly suggest that with IAA precursor availability, most likely tryptophan, in root exudates, IAA biosynthesis by the fungus itself might increase fungal ramification in intercellular spaces of root cortical cells, ultimately increasing mycorrhization speed and efficiency. Up to this point, the involvement of IAA in *T. vaccinum*-spruce ectomycorrhiza was still largely speculative. Thus, we tested the function of IAA in ectomycorrhizal symbiosis by exogenously applying the phytohormone to *T. vaccinum*-spruce ectomycorrhiza cultures *in vitro*. Indeed, the phytohormone increased ectomycorrhiza formation as shown by significantly higher Hartig' net formation in cultures supplemented with 100 µM IAA. It is suggested that the growth and ramification effects of IAA on *T. vaccinum* observed *in vitro* represents a new mechanism involved in mycorrhiza morphogenesis. Interestingly, the results also suggest that IAA acts as a signal in the fungal-plant interaction in ectomycorrhizal symbiosis.

The isolation and characterization of *T. vaccinum ald1*, the first ALDH-encoding gene to be studied in mycorrhizal fungi, will form the basis of future work on the role of IAA in ectomycorrhizal symbiosis, communication of the symbiotic partners, and the isolation of other *ald* paralogs in order to fully resolve the aldehyde and alcohol stress torelance in ectomycorrhiza. The use of ATMT in functional gene analysis, demonstrated for the second time in ectomycorrhizal fungi, shows that the method has the potential for investigating gene functions in *T. vaccinum* as well as in other ectomycorrhizal fungi.

ZUSAMMENFASSUNG

Das funktionelle Verständnis der Ektomykorrhiza entwickelt sich aufgrund der wenigen molekularen Untersuchungen dieser Symbiose nur vergleichsweise langsam. In dieser Arbeit wurde die mögliche Rolle einer spezifisch pilzlichen Aldehyd-Dehydrogenase (ALDH) in der Symbiose zwischen dem zu den Basidiomyzeten gehörenden Ektomykorrhiza-Pilz *Tricholoma vaccinum* und seiner kompatiblen Wirtspflanze, der Fichte (*Picea abies*), untersucht. Auch der Einfluss von Indol-3-Essigsäure (IAA) auf die Ektomykorrhizabildung, die bereits seit längerer Zeit in der Literatur kontrovers diskutiert wird, wurde detailliert untersucht.

Zunächst wurde das in der Ektomykorrhiza spezifisch exprimierte Gen *ald1* aus *T. vaccinum* isoliert und charakterisiert. Die Sequenzanalyse zeigte, dass der offene Leserahmen von 16 Introns unterbrochen ist. Aus der Sequenz konnte das Protein Ald1 mit 502 Aminosäuren abgeleitet werden. Das vorhergesagte Molekulargewicht konnte durch Westernblot-Analyse mit 53 kDa bestätigt werden. Um die evolutionäre Beziehung zwischen den ALDHs dieser Enzymfamilie zu untersuchen, wurde in dieser Arbeit ein Alignment von Ald1 mit anderen pilzlichen ALDHs erstellt. Insgesamt konnten 53 spezifisch pilzliche ALDHs aus allen Hauptgruppen der Pilze verglichen werden. Die auf Bayes'scher Interferenz basierende phylogenetische Untersuchung ergab, dass, mit Ausnahme der Chytridiomycota, die pilzlichen ALDHs klare taxonomische Gruppen im Phylogramm bilden. Diese sind im Laufe der Evolution durch zwei Duplikationsereignisse entstanden, so dass multiple ALDH-Paraloge vorliegen. Dies betrifft insbesondere die höheren Pilze und trifft auch für *T. vaccinum* zu.

Stress-Response-Elemente (STREs) wurden in der Promotorregion des *ald1*-Gens identifiziert, was nicht überrascht, da eine wahrscheinliche Funktion im Abbau von Aldehyden liegt, die in der Zelle entgiftet werden müssen. Eine Induktion dieses Gens durch Stressfaktoren ist daher naheliegend. Daher wurde im Folgenden ie Induktion von *ald1* durch Aldehyde und Alkohol mittels Real time-PCR untersucht. Eine signifikant erhöhte Genexpression wurde bei Wachstum mit 0,1 mM Indol-3-Acetaldehyd (IAAld), 0,1 mM Benzaldehyd und 0,01% Ethanol festgestellt.

Um die biologische Funktion von *ald1* in *T. vaccinum* zu charakterisieren, wurde es homolog im Pilz über *Agrobacterium tumefaciens*-vermittelte Transformation (ATMT) überexprimiert. Die funktionelle Analyse zeigte, dass Ald1-überproduzierende Transformanten deutlich

weniger anfällig für Ethanolstress waren als der Wildtypstamm. Diese Resultate demonstrieren die Fähigkeit der ALDH, durch Ethanol erzeugten Stress zu umgehen; eine entscheidende Funktion in Ektomykorrhiza-Habitaten.

Die Induktion der *ald1*-Expression durch IAAld deutete darauf hin, dass sie zumindest teilweise an der Produktion der Indol-3-essigsäure (IAA) beteiligt sein könnte. Daher wurde die IAA-Biosynthese in *T. vaccinum* untersucht. Mit Hilfe des Nachweises durch GC-MS konnte gezeigt werden, dass der Pilz nur in Anwesenheit von Tryptophyn oder IAAld messbare Mengen von IAA produziert, vermutlich über den Tryptophan-Indolpyruvat-IAAld-IAA-Biosyntheseweg. Die Synthese von IAA durch den Pilz machte eine Charakterisierung der Auswirkungen exogener IAA-Gaben zur Charakterisierung der Wirkung von IAA auf *T. vaccinum* nötig. Die exogene Zugabe von IAA zu *T. vaccinum*-Kulturen erhöhte signifikant das Pilzwachstum und die Hyphenverzweigung. Diese Wirkung wurde durch Zugabe des IAA-Transportinhibitors TIBA aufgehoben. Zusammen genommen deuten die Resultate darauf hin, dass mit verfügbaren IAA-Vorstufen wie Tryptophan in Wurzelexsudaten, dieser Pilz in der Lage ist, IAA zu produzieren. Dies wiederum führt zu erhöhter Hyphenverzweigung in den Interzellularräumen der Rindenzellen und somit zur schnellen und effektiveren Mykorrhizierung. Bisher war der Einfluss von IAA auf Ektomykorrhiza spekulativ, so dass hier eine funktionelle Verbindung mit der Synthese eines Pflanzenhormons und der Bildung der Symbiose erstmals gezeigt werden konnte. Um die Rolle von IAA zu bestätigen, wurde das Phytohormon *in vitro* zu Co-Kulturen von *T. vaccinum* und Fichte gegeben, wobei 100 µM IAA zu einer verstärkten Ausbildung des Hartig' schen Netzes im Vergleich zu Kontrollkulturen führte. Damit konnten die *in vitro* beobachteten Wachstums- und Verzweigungsffekte von IAA auf *T. vaccinum* mit der Expression des Gens *ald1* verknüpft werden und ein funktioneller Zusammenhang zwischen IAA und der Ektomykorrhizaentwicklung hergestellt werden.

Die Isolierung und Charakterisierung des Gens *ald1* aus dem Ektomykorrhizapilz *T. vaccinum* bietet die Grundlage für zukünfige Arbeiten wie beispielsweise die Isolierung anderer *ald*-Paraloge, um so die Aldehyd- und Alkohol-Stresstoleranz in der Ektomykorrhiza aufzuklären. Die hier gezeigte Verwendung der ATMT in der funktionellen Genanalyse öffnet neue Möglichkeiten nicht nur für die Analyse weiterer *ald*-Paraloge, sondern auch zur funktionellen Analyse anderer Gene in *T. vaccinum* und in anderen Ektomykorrhiza-Pilzen.

1. INTRODUCTION

1.1. Mycorrhiza (general overview)

The term mycorrhiza conventionally refers to a mutualistic symbiosis between plant roots and filamentous fungi. However, it should be cautiously used since some experiments have shown that not all the mycorrhizal associations are mutualistic (Smith and Read, 1997) in terms of nutrient cost-benefit ratio. It is estimated that over 90% of terrestrial plants naturally form mycorrhiza with soil fungi (Cairney, 2000). Ever since Frank (1885) recognized the essential aspects of the association, it has been suggested that in this mutualistic symbiosis, there is a bidirectional flow of nutrients in which the fungus mobilizes and supplies inorganic nutrients to the plant, and the plant primarily supplies carbohydrates to the fungus. Mycorrhizal associations improve plant/crop growth, and this becomes relevant in environments which are frequently flooded or drought prone, where non-mycorrhized plants would not survive due to increased stress. Furthermore, mycorrhizal associations increase resistance of plants to pathogens (Farquhar and Peterson, 1991; Smith and Read, 1997; Pozo and Azcon-Aguilar, 2007; Zhang *et al*., 2008) and support plant growth on poor soils (Smith and Read, 1997).

Mycorrhizal associations are broadly divided, on the basis of, but not limited to, their fungal partners and structures formed, into vesicular arbuscular (VA) endomycorrhiza, ectomycorrhiza and ericoid mycorrhiza (Smith and Read, 1997). The VA mycorrhiza involves interaction of aseptate fungi in the order Glomales of the zygomycota with most herbaceous and woody angiosperm and gymnosperm plants. This type of mycorrhiza is the most ancient of all mycorrhizal types, as shown by fossil records and DNA sequences of living members; this could have happened about 450-500 million years ago (Smith and Read, 1997; Cairney, 2000). Subsequently, as the organic matter of ancient soils increased, ectomycorrhiza (about 200 million years ago), and later ericoid mycorrhiza (about 100 million years ago) evolved (Cairney, 2000). Ectomycorrhiza is formed between tree or woody angiosperms and gymnosperms, and the majority of ectomycorrhizal fungi belonging to the subphylum basidiomycotina (Kendrick, 1992) while ericoid mycorrhiza are formed by ascomycetes on plants of the order Ericales (Smith and Read, 1997). Whereas endomycorrhizae have convincingly been studied, perhaps because most of them are associated with annual crops in agriculture, much remains unknown about ectomycorrhizae, especially at the molecular level. Still, the latter associations play a central role in ecosystem functioning and stability since they are involved in agriculture and forestry.

1.2. Ectomycorrhiza

1.2.1. Structure and function

Although ectomycorrhizal associations have been known to exist for a very long time, it is only in the recent past that we began to understand them; this development was undoubtedly aided by *in vitro* systems that have been developed, and to a lesser extent, the wealth of the *in vivo* knowledge generated by morphotyping and sequencing of ITS region of fungal rDNA. In this mycorrhizal type, the fungus and plant interact to differentiate morphological features typical of this association. Initially, the fungus grows around the plant root forming a network of hyphae called mantle, from which some hyphae penetrate the root to grow intercellularly between cortical cells to form the Hartig' net, which acts as a surface for exchange of nutrients between the two partners (Smith and Read, 1997; Fig. 1 A). Interestingly, these features can be observed, albeit at low frequencies, in axenic cultures without amending minimal medium with glucose or sucrose to increase fungal growth, as has been described by other groups, leading to development of realistic *in vitro* systems that reflect natural settings (Asiimwe *et al*., 2010).

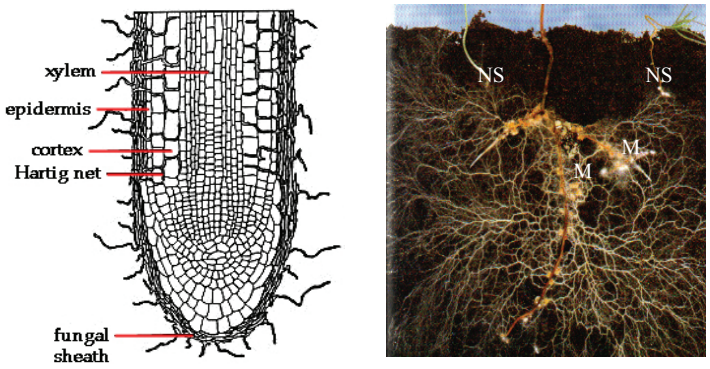


Fig. 1. A: Schematic longitudinal section of a plant root showing ectomycorrhizal features (http://biology.uwsp.edu); B: An extensive mycelial and rhizomorph development by *Suillus bovinus* in symbiosis with a seedling of *Pinus sylvestris* (adapted from Smith and Reed, 1997). Interesting to note is the extraradical fungal system, emanating from fungal mantle (M), which has already mycorrhized new germinating seedlings (NS).

Morphotyping studies indicate that ectomycorrhizal fungi extend their growth from mantle to the bulk soil, exploring long distances with the help of an extensive network of individual hyphae or organized hyphal aggregates called rhizomorphs, which emanate from the mantle (Baier *et al*., 2006; Agerer, 2001; Smith and Read, 1997), as depicted in Fig. 1B. This extensive hyphal network facilitates absorption of nutrients, either in dissolved form or mobilized from organic to inorganic sources by extracellular enzymes, sequestered through

excretion of organic acids, and water uptake, which helps mycorrhized plants to better exploit unfavourable ecological niches.

1.2.2. Ectomycorrhizal fungi

The majority of fungi that form ectomycorrhizae are basidiomycetes, although a few ascomycetes and a few species of zygomycetous fungi in the genus *Endogone* are known to form this type of association (Smith and Read, 1997). It is not surprising, therefore, that a larger number of fungal species, as compared to VA mycorrhiza, form ectomycorrhiza. It has been estimated, based on field observations, that about 5000-6000 species of fungi form ectomycorrhizas (Molina *et al.,* 1992). Of course, this ought to be verified experimentally by synthesizing ectomycorrhizae *in vitro* to confirm the field estimates. Observations of plant-sporocarp associations suggest that the majority of ectomycorrhizal fungi have a broad host range. For example, species such as *Amanita muscaria*, *Cenococcum geophilum*, *Hebeloma crustuliniforme*, *Laccaria laccata*, *Pisolithus tinctorius* and *Thelephora terrestris* are distributed world wide on a very wide range of plants (Smith and Read, 1997). However, some fungi show a narrow host range or, in fact, form mycorrhiza only with a single plant host specie. This is the case for *Tricholoma vaccinum*, which forms natural ectomycorrhiza only with spruce. In total, about 200 species of the genus *Tricholoma*, most of which form ectomycorrhiza, are now known to exist (Kirk *et al*., 2008). Interestingly, Ogawa (1985) observed that the "ectomycorrhiza" formed between *Tricholoma matsutake* and *Pinus spp*. lacked a Hartig' net, and that the association was, actually, parasitic. Because of this, Smith and Read (1997) discouraged the use of the term "ectomycorrhiza" to describe this specific association. However, the formation of true ectomycorrhiza by this fungus was later demonstrated by Yamada *et al.* (1999) by using bottle cultures of *T. matsutake-Pinus densiflora* ectomycorrhiza *in vitro*. Their success was, however, probably due to differences in infectivity or pathogenicity with isolates used in other different studies. Nonetheless, *T. matsutake* has attracted tremendous research attention, primarily because the fungus is one of the most popular edible mushrooms. Although the genome of *T. vaccinum* has not been sequenced, the availability of a sequenced genome of its close relative, *Laccaria bicolor,* the first ectomycorrhizal fungus to be fully sequenced (Martin *et al*., 2008), will facilitate its investigation.

1.2.3. Molecular plant-fungal interaction

Much is now widely known about the morphology and function of ectomycorrhizae and the specificity of the interaction. In fact, over the past decades, the theme of "ectomycorrhiza structure" has become obvious. However, the fundamental question about what drives the interaction of plant and mycobiont, and the subsequent differentiation of the observed structures, remain unanswered. This can be resolved by investigating signals, together with their regulation, involved in the communication between the mycobionts and their plant partners during mycorrhiza development. Unfortunately, such studies are rare, although molecular basis of ectomycorrhizal interaction is undoubtedly central to its understanding. Also, in order to better utilize ectomycorrhizal fungi in biotechnology, thorough knowledge about their signal production and perception during ectomycorrhizal interaction is necessary.

The morphological and physiological changes differentiated in ectomycorrhizae have been shown, by a few recent studies, to be accompanied by changes in gene transcript pattern. Genes which are differentially expressed in ectomycorrhizae have been identified in eucalypt-*Pisolithus* ectomycorrhiza (Tagu *et al*., 1993; Tagu and Martin, 1995; Duplessis *et al*., 2005), the first type of ectomycorrhiza to receive intensive research attention in this field (Martin and Tagu, 1995). This is still regarded as a model system in ectomycorrhizal studies. In another interaction, differential display was carried out to characterize the host-specific *Tricholoma*-spruce ectomycorrhiza (Krause and Kothe, 2006). Although this approach yielded several genes hypothesized to be involved in the interaction, physiological functions or mechanisms regulating expression have not yet been elucidated. This is compounded by the fact that most of the ectomycorrhizal fungi, mainly basidiomycetes, are not amenable to laboratory experimentation. Nevertheless, recent demonstrations that some of these fungi can be transformed present an opportunity to unravel the possible biological functions of genes identified in differential display studies in ectomycorrhiza establishment and maintenance.

1.3. Aldehyde dehydrogenases as metabolically versatile enzymes

1.3.1. Metabolic roles of aldehyde dehydrogenase

ALDHs have been identified, in all phyla, to play various roles in metabolism, primarily, but not limited to, metabolic roles resulting from oxidation of different aldehydes to their corresponding carboxylic acids. Although these reactions can also be catalyzed by aldehyde oxidase and xanthine oxidase, ALDH is the major enzyme (Panoutsopouluos and Beedham, 2005). The presence of these enzymes across all phyla suggests that they play critical roles in

metabolism of living organisms. Perozich *et al* (1999) phylogenetically investigated the ALDH relationships by comparing 145 ALDH sequences across all phyla, and revealed several conserved residues and motifs in the enzyme superfamily, suggesting common catalytic activities. The authors indicated that a typical ALDH should have between 450 and 520 amino acids, and that it possesses 10 highly conserved motifs, which cluster around the active site of the enzyme (Fig. 2). A high variability in substrate specificity occurs in ALDHs, with some being highly specific for a limited range of substrates while others show broad substrate specificity. This forms the basis of their classification into gene families comprising the ALDH super family. Although 13 ALDH families were earlier reported (Perozich *et al*., 1999), this has been updated to 20 families (Sophos and Vasiliou, 2002), and the number is likely to further increase as more genomes are sequenced. All ALDHs require either NAD or NADP as a cofactor, as indicated by many authors, although some ALDHs have been shown to bind either cofactor, albeit with different affinities.

Fig. 2. Proposed secondary structure of aldehyde dehydrogenase showing highly conserved motifs, which cluster around the enzyme active site (gray region); the structure of rat cytosolic class 3 ALDH was generated by Sayle and Milner-White (1995).

ALDH is normally regarded as an enzyme of detoxification (Jacoby and Ziegler, 1990) that most likely performs a dual role: metabolism of physiological compounds i.e. metabolic intermediate aldehydes, and active detoxification of exogenous aldehydes, all of which are normally toxic at low levels because of their chemical reactivity. Endogenous physiologically-derived aldehydes arise from metabolism of amino acids, biogenic amines, carbohydrates, vitamins, steroids, and lipids while xenobiotics (including drugs) are a major source of exogenous aldehydes (reviewed by Lindahl, 1992).

Detoxification of aldehydes is closely linked to that of alcohols because ALDH is the second enzyme in alcohol metabolism, the first being alcohol dehydrogenase (ADH). In alcohol metabolism, an alcohol is oxidized to an aldehyde by ADH, which is later on also oxidized to the corresponding carboxylic acid by ALDH (Fig. 3). This positions ALDH as a central

enzyme in detoxification since both aldehydes and alcohols are abundant in nature, especially when an organism is periodically exposed to stress, which is normally the case. The end products of alcohol and aldehyde metabolism, though not depicted in the scheme (Fig. 3), are non-toxic usable carbohydrates. This would explain, at least partly, why some fungi are able to utilize these rather toxic compounds as alternative nutrient sources. However, it would come at an energy cost, since an organism would have to increase production of ALDH and ADH, a phenomenon that would lead to a metabolic disadvantage.

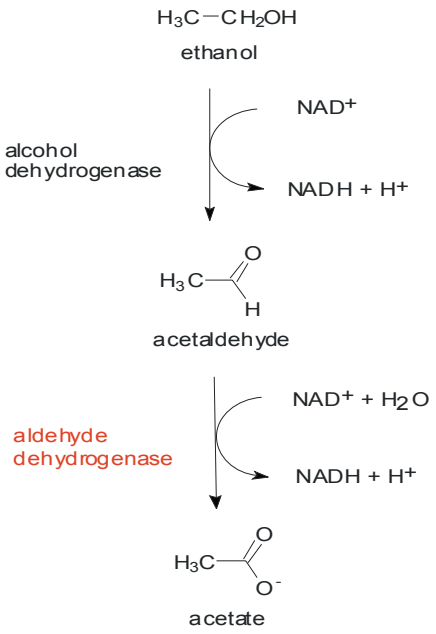


Fig. 3. A simplified scheme illustrating ethanol catabolism: Ethanol is sequentially oxidized to acetaldehyde and acetate, catalyzed by ADH and ALDH respectively, by utilizing NAD as a cofactor (Scheme adapted from Krause, 2005).

Apart from being involved in detoxification of alcohols and aldehydes, ALDH is a major enzyme that catalyzes production of the phytohormone IAA from indole-3-acetaldehyde (Cooney and Nonhebel, 1989; Basse *et al*., 1996; Tam and Normanly, 1998; Fedorova *et al*., 2005; Spaepen *et al*., 2007; Reineke *et al*., 2008), the last step in tryptophan-dependent IAA biosynthesis, using the indole-3-pyruvic acid pathway (Woodward and Bartel, 2005). While IAA is traditionally known as a plant hormone that mediates plant growth and development, it has been observed to play other important metabolic roles mediating plant-microbe interaction, whether beneficial or pathogenic, and microbe-microbe interaction.

1.3.2. Regulation of aldehyde dehydrogenase

The fact that aldehydes are highly reactive, mainly toxic in low concentrations, and are generated from limitless numbers of endogenous and exogenous sources, demands a tight regulation of the oxidizing enzyme, ALDH, for survival. While it is well established that *ald* genes are stress-inducible, the real inducers of the genes are most likely aldehydes, which mainly, but not solely, accumulate from reactions of reactive oxygen species (ROS) that excessively accumulate in cells after exposure to stress, with lipids and proteins. The evidence of this suggestion mainly comes from the well studied ethanol utilization pathway in *Aspergillus nidulans*, although there is also evidence from other organisms. In *A. nidulans*, ALDH and ADH are co-regulated, at the transcription level, by the so called *alc* regulon. In this *alc* gene system, the pathway-specific transcriptional activator AlcR mediates the induction of *aldA,* the aldehyde dehydrogenase-encoding gene, and ADH-encoding gene (*alc*) in the presence of either ethanol or acetaldehyde as co-inducing compounds (Fillinger *et al*., 1995; Felenbok *et al*., 2001; Flipphi *et al*., 2001) (Fig. 4).

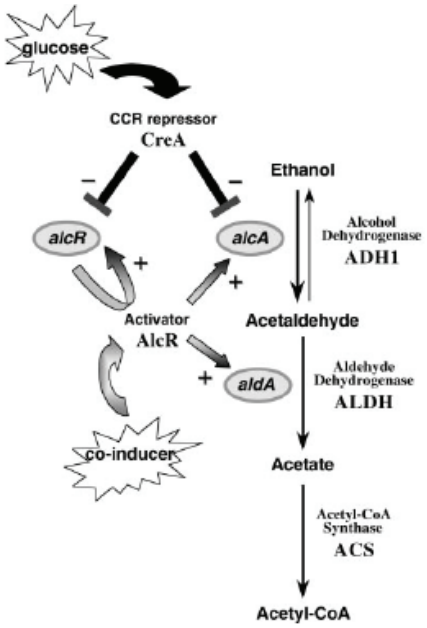


Fig. 4. Schematic diagram showing the regulation of ethanol catabolic pathway. AlcR, which is subject to positive autoregulation (curved arrow), activates the alcohol dehydrogenase- and aldehyde dehydrogenase-encoding genes (thick arrows) in presence of a co-inducer. The CreA repressor, in the presence of glucose, directly represses the structural genes (adapted from Flipphi *et al*., 2001).

Apart from ethanol and acetaldehyde, other inducers of the *alc* system e.g. amino acids and other aliphatic alcohols have been identified, but it was concluded that the real physiological inducers are aldehydes, and that all other inducer compounds have to first be converted to their corresponding aldehydes (Flipphi *et al*., 2001; Flipphi *et al*., 2002; Flipphi *et al*., 2003).

The *alc* gene system is negatively controlled by the general catabolite repressor CreA in the presence of glucose as a repressing carbon source. The physiological induction of alcohol metabolism by aldehydes, in a tight co-regulation of ALDH and ADH, and given that the former also participates in detoxification of other non-alcohol derived aldehydes, positions ALDH as a critical enzyme in metabolism.

1.3.3. Possible roles of aldehyde dehydrogenase in ectomycorrhiza

In nature, plants inhabit environments that are occasionally water-logged or flooded. Under these anaerobic conditions, glycolysis and alcoholic fermentation is necessary for the plant's survival. Unfortunately, concentrations of aldehydes are dramatically increased in the process, which then requires an ALDH-aided detoxification mechanism. 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). It has been shown that mycorrhiza increases flood tolerance of plants, mainly through suppression of toxic products of anaerobic respiration like ethanol (Osundina, 1998; Rutto *et al*., 2002), and to a lesser extent through increased nitrogen acquisition, among others (Neto *et al*., 2006). Although these observations were made on VA-mycorrhiza, they might be extrapolated to suggest the same role in ectomycorrhiza, considering many other shared physiological roles between these mycorrhizal types.

Mycorrhiza-enhanced flood tolerance by detoxification of anaerobic respiration products would suggest a direct role of a fungal ALDH in mediating stress effects derived from flooded or water-logged conditions. However, this is still a matter of speculation because there were no *ald* genes isolated to that effect, another reminder of the less molecular characterization in mycorrhizal studies. Nevertheless, evidence of a possible role of a fungal ALDH in ectomycorrhiza development and maintenance has recently started emerging, as shown by the study that identified a fungal *ald* as one of the genes that were differentially expressed in *T. vaccinum*-spruce ectomycorrhiza (Krause and Kothe, 2006). In fact, a total of three *ald* partial sequences from *T. vaccinum*, which cluster, in a phylogenetic tree, with other basidiomycete ALDHs have been reported (Krause, 2005; Asiimwe *et al*., 2010). Also, since an ALDH was shown to be involved in production of IAA in a basidiomycete, *Ustilago maydis* (Basse *et al*., 1996; Reineke *et al*., 2008), this could also be true for basidiomycete ectomycorrhizal fungi. Although still controversial, IAA has been suggested to increase mycorrhization efficiency.

1.4. Indole-3-acetic acid in metabolism

1.4.1. Metabolic roles, biosynthesis, and regulation of indole-3-acetic acid

IAA, which is recognized as the key auxin in most plants (Woodward and Bartel, 2005), is a critical plant hormone that modulates different developmental processes such as general root and shoot architecture, tropic responses to light and gravity, organ patterning, vascular development and growth in tissue culture (Davies, 1995), all of which are fundamental to plant growth and development. However, IAA seems not only to be active in growth promotion of plants, but also of fungi because it has been shown that fungal growth was increased as a consequence of exogenous IAA application (Nasim *et al*., 2004). In addition, some fungi (Podila, 2002) and bacteria (Spaepen *et al*., 2007) are able to produce IAA. Predominantly a plant hormone, it is not clear how microbes developed the trait of IAA production during their evolution, but it, most probably, could have been developed as a signal to manipulate the host metabolic and defense systems, since most microbes producing IAA are plant-associated. Whereas there is convincing evidence to support this hypothesis in bacteria (Spaepen *et al*., 2007), where bacterial IAA was shown to modulate plant IAA signaling or host defense responses, the physiological role of fungal IAA in host interaction is still largely speculative. Furthermore, IAA was shown to mediate plant growth promotion by plant growth-promoting rhizobacteria (PGPR) (Xie *et al*., 1996; Bashan and Holguin, 1997), and nodulation in rhizobium-legume symbiosis (Mathesius *et al*., 1998).

Although a growth-promoting hormone, IAA is toxic at high levels, and is also an unstable compound, which makes it plausible to suggest that IAA metabolism may be tightly controlled. Indeed, this seems to be the case, as only small amounts of free IAA in plants, bacteria or fungi are present, most of it existing in form of conjugates. These conjugates serve diverse roles, like transport, storage and protection of IAA from enzymatic degradation (Spaepen *et al*., 2007), but also homeostatic control of its levels in the cell, and allow its catabolism (Östin *et al*., 1998; Seidel *et al*., 2006). Overall, cellular IAA concentration can be controlled at multiple levels, such as biosynthesis, conjugation, deconjugation, degradation, and intercellular transport (Vanneste and Friml, 2009) to maintain the physiologically relevant levels. Despite the fact that there are elegant studies demonstrating that high IAA concentration may be important for some physiological processes like organ initiation (Dubrovsky *et al*., 2008), the so-called auxin maxima, differential IAA distribution seems to be the key to initiation of many IAA- dependent processes.

The tight control of IAA metabolism would also imply tight regulation of its biosynthesis. Despite the fact that the physiological roles and importance of IAA are clearly understood, its biosynthetic pathways have, surprisingly, not been fully elucidated. This is, perhaps, because of the existence of multiple IAA biosynthetic pathways, functional redundancies among the participating enzymes, and existence of most of IAA in conjugated form, as reviewed by Eckardt (2001). Thus, until now, there is no single, complete IAA biosynthetic pathway that has been identified, although various pathways have been proposed, on the basis of a few biosynthetic genes cloned and detection of biosynthetic intermediates. Furthermore, the pathways are proposed from a pool of different studies and systems, mainly in plants and less for microbes. Broadly, two main IAA biosynthetic pathways have been proposed: tryptophan-dependent and tryptophan-independent pathways (Fig. 5). The tryptophan-independent pathway was proposed by Normanly *et al* (1993), on the basis of *Arabidopsis* mutants with reduced levels of tryptophan synthase, which showed increased amounts of IAA conjugates. Unfortunately, their proposal was refuted when it was realized that the increase in IAA conjugates was, in fact, a result of non-enzymatic degradation of indole-3-glycerophosphate, the IAA (and tryptophan) precursor that hyper-accumulates in the mutants (Müller and Weiler, 2000).

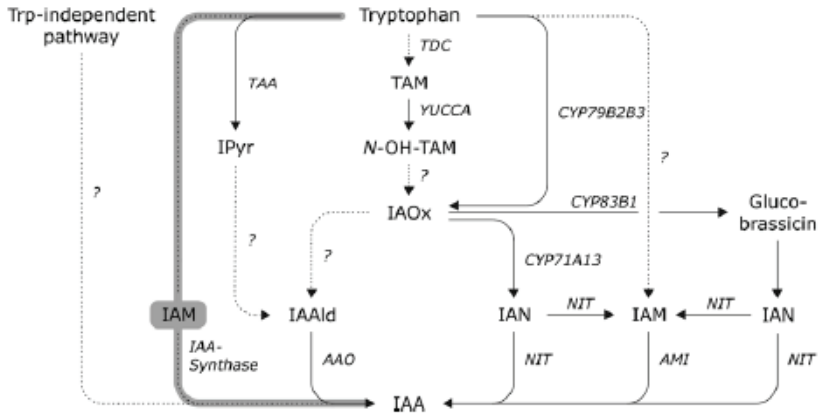


Fig. 5. A scheme showing the proposed IAA biosynthetic pathways found in plants and microorganisms. The dashed lines indicate assumed reaction steps for which the predicted genes/enzymes have not been identified (adapted from Pollmann *et al*., 2009). Tryptophan-independent pathway assumes direct IAA production from non-tryptophan precursors. In tryptophan-dependent pathway, IAA is produced through indole-3-acetamide (IAM), indole-3-pyruvate (IPyr), indole-3-acetaldehyde (IAAld), tryptamine (TAM), indole-3-acetaldoxime ((IAOx), and indole-3-acetonitrile (IAN) as the main biosynthetic intermediates.

As a matter of fact, the tryptophan-independent pathway is now widely disputed since, so far, neither intermediates nor biosynthetic enzymes have been presented, putting tryptophan at the center stage as the primary precursor in IAA biosynthesis. In the tryptophan-dependent

pathway, IAA production proceeds *via* four "hallmark" intermediates, i.e. indole-3-acetamide (IAM), indole-3-pyruvate (IPyr), tryptamine (TAM), and indole-3-acetaldoxime (IAOx) (Woodward and Bartel, 2005; Pollmann *et al*., 2009). It should, however, be emphasized that most of the knowledge about the pathways is from plants. Nevertheless, as reviewed in Spaepen *et al* (2007), all pathways, excluding IAOx, have also been proposed in bacteria. Although many reports have documented production of IAA by fungi, the biosynthetic pathways are largely unknown. Most of what is currently known about IAA biosynthesis in fungi comes from the basidiomycete smut fungus *Ustilago maydis*, which has served as a model organism in these studies. In this regard, it has been proposed that IAA biosynthesis in *U. maydis* proceeds from tryptophan, *via* IPyr and IAAld (Basse *et al*., 1996; Bölker *et al*., 2008; Reineke *et al*., 2008; Zuther *et al*., 2008). Although TAM feeding experiments showed that *U. maydis* is able to convert TAM to IAA (Basse *et al*., 1996; Reineke *et al*., 2008), the downstream intermediates are not known, in which case the TAM pathway in this fungus remains unresolved. Furthermore, IPyr and IAM pathways have also been proposed in *Colletotrichum gloeosporioides* and *C. acutum* (Robinson *et al.*, 1998; Chung *et al*., 2003). So far, no investigation on IAA biosynthetic pathways has been reported for ectomycorrhizal fungi.

1.4.2. Role of indole-3-acetic acid in ectomycorrhiza development

Auxin was proposed, in the so-called 'auxin theory' of Slankis (1973), as one of the agents contributing to the regulation of ectomycorrhiza development, by stating that mycorrhiza formation was regulated through the controlled production of auxins by ectomycorrhizal fungi. Ever since that time, the involvement of IAA in ectomycorrhiza development and maintenance became controversial, which, unfortunately, seems to remain the case until today. A strong argument in support of Slankis' theory first came in 1994, when Gay *et al*., (1994) used IAA-overproducing transformants of *Hebeloma cylindrosporum* on *Pinus pinaster* seedlings to show that fungal IAA increases ectomycorrhiza formation. In fact, a cytological confirmation to these observations was made by using one of the mutants (Gea *et al*., 1994), where it was observed that the IAA-overproducer formed ectomycorrhiza that was characterized by a more highly developed Hartig' net, up to seven layers of hyphae in width, which reached the endodermis (Fig. 6). However, these two studies, fascinating as they are, indicated that there was no correlation between the IAA synthesizing ability of the transformants and their mycorrhizal activity, and general plant growth.

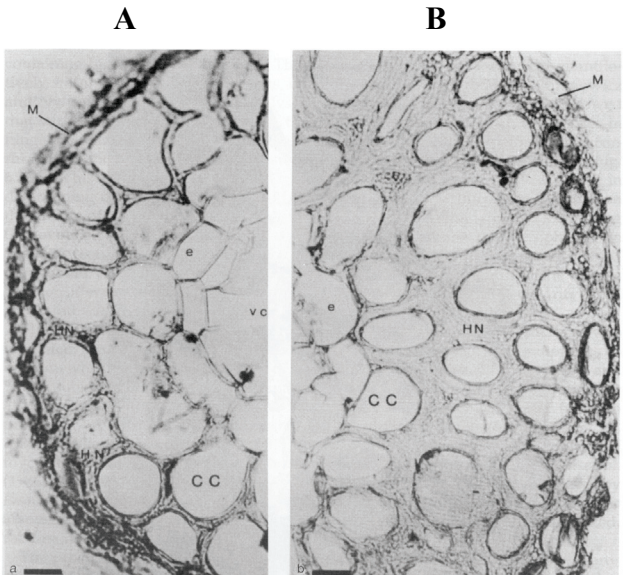


Fig. 6. Cross sections of *H. cylindrosporum/ P. pinaster* ectomycorrhiza showing wild type (**A**) and IAA-overproducing (**B**) fungal colonization (From Gea *et al*., 1994). Note that the IAA-overproducing transformant formed more extensively developed Hartig' net (HN) between cortical cells (CC) than the wild type.

Nevertheless, the studies suggest that fungal-derived IAA might trigger morphogenetic changes in host roots as well as the fungal partner during ectomycorrhiza formation. Exactly how this would happen is not understood, although, as hypothesized in a review by Podila (2002), fungal IAA could function as a signal to trigger transcription of auxin responsive genes, as supported by up-regulated genes in *P. pinaster* in response to colonization by *H. cylindrosporum* and external IAA application (Charvet-Candel *et al*., 2002; Reddy *et al*., 2003), which would in turn trigger a cascade of molecular events in plant roots leading to formation of mycorrhiza. Many ectomycorrhizal fungi have been shown to produce IAA, but mainly in presence of tryptophan as the precursor, although the biosynthetic pathways are not known. However, the role of IAA in differentiating ectomycorrhiza features remain controversial (reviewed in Barker and Tagu, 2000; Podila, 2002). Whereas Gay *et al*., 1994 and Gea *et al*., 1994 convincingly demonstrated that IAA is involved in ectomycorrhiza development by using the elegant IAA-overproducing transformants of *Hebeloma cylindrosporum*, which was later confirmed by using fungi with different IAA-synthesizing abilities and exogenous application of IAA and its inhibitors by different authors (for example Rudawska and Kielszewska-Rokicka, 1997; Niemi *et al*., 2002; Rincón *et al*., 2003; Herrmann *et al*., 2004), other authors indicated otherwise (Horan, 1991; Wallander *et al*., 1992; Wallander *et al*., 1994; Hampp *et al*., 1996). While tryptophan availability in root exudates is not expected to be at the level of concentrations used in various experiments to test the ability of ectomycorrhizal fungi to produce IAA, it could nonetheless be sufficient to

trigger the increased biosynthesis of IAA by ectomycorrhizal fungi in the symbiotic interaction.

Understanding involvement of fungal IAA in ectomycorrhizal development can not be complete without investigating the possible function of the phytohormone in the biology of ectomycorrhizal fungi. Judging from reports on the role of IAA in ectomycorrhiza, it is plausible to suggest that, although fungal IAA plays a role in ectomycorrhiza development, the effect is most probably a contribution to that of other fungal and plant factors, which are also involved in regulation of ectomycorrhiza formation.

1.5. Fungal transformation

1.5.1. Transformation (general overview)

Genetic transformation systems represent an essential factor in advancement of functional gene analysis or engineering organisms to express desirable traits. Specifically for the latter, commercial production of enzymes or important metabolites, and the production of genetically engineered organisms for broader application in agriculture and forestry is possible. Through genetic transformation, genomes of organisms can be manipulated, correlating *in vitro* studies of purified DNA with biological consequences *in vivo*. Apart from the yeast *Saccharomyces cerevisiae* and a few industrially important filamentous fungi, functional gene analysis in filamentous fungi has been hampered by more complicated, and often time-consuming, inefficient transformation methods. Yet, with the current genomics revolution, where increasing number of fungal genomes are completely sequenced, need arises for development of efficient transformation systems to unravel the biological functions for DNA sequences that have been determined in large scale sequencing efforts. As broadly stated for microorganisms (Ruiz-Díez, 2002), molecular genetic manipulation of fungi requires the development of efficient transformation systems that include: (1) introducing exogenous DNA into recipient cells; (2) expression of genes present on transforming DNA; and (3) stable maintenance and replication of the inserted DNA, leading to expression of desired phenotypic traits.

1.5.2. Transformation methods

Three decades after the first report on successful transformation of *S. cerevisiae* (Hinnen *et al*., 1978), efficient transformation systems have been developed for only a few dozen

filamentous fungi. Whereas a number of fungal transformation methods exist, the single most important setback to genetic transformation of a multitude of fungi is that the methods are not cross-cutting and so, new transformation systems need to be developed for individual fungi, which is not trivial for many of them. Moreover, once a transformation system has been successfully developed, there may still be barriers to use it for analysis of gene function. It is, therefore, imperative to carefully choose a transformation method that is most suitable for the genetic engineering strategy at hand. The most promising methods that have been used in fungal transformation are protoplast transformation, biolistic transformation, electroporation and *Agrobacterium*-mediated transformation (AMT). The features as well as advantages and disadvantages of these transformation methods are reviewed in Meyer (2008) and others (for example Ruiz-Díez, 2002; Michielse *et al*., 2005; Weld *et al*., 2006). AMT, which relies on the ability of *A. tumefaciens* to transfer the T-DNA region of a binary vector carrying transforming cassettes, which is then integrated into the recipient's genome, has been singled out as a method of choice. This is, because the method has superior features which include the ability to transform a variety of fungal tissues, high efficiency of transformation, increased frequency of homologous recombination and low copy number (mostly single copy) of inserted T-DNA per genome. In fact, AMT has been described as an efficient method for transformation of fungi that were recalcitrant to other transformation methods (Michielse *et al*., 2005). The predominantly single DNA insertion into genomes by AMT greatly simplifies the task of demonstrating that the tagged gene represents the mutation responsible for the phenotype observed in the transformants.

1.6. Aims of the study

Investigating the molecular basis of ectomycorrhizal interaction is central to its understanding. Our knowledge on this subject is rather scanty, as most reports have been on morphology and physiology of the interaction. The main aim of this study was to investigate the molecular mechanisms that drive *Tricholoma vaccinum*-spruce (*Picea abies*) ectomycorrhizal interaction. An earlier study on this ectomycorrhiza had identified an aldehyde dehydrogenase encoding gene (*ald1)* as one of the genes that were differentially up-regulated in the mycorrhizal interaction (Krause, 2005; Krause and Kothe, 2006). This gene, expected to play a crucial role in ectomycorrhiza by the virtue of its metabolic roles, was fully isolated and characterized, specifically with regard to transcriptional regulation and functional

characterization. Among potential inducers tested were ethanol as well as different aldehydes, including IAAld.

The notion that fungal IAA is involved in ectomycorrhiza establishment and maintenance is still controversial, as ever, probably because IAA regulation is tight or simply because some important aspects, like the effect of IAA on fungal biology have not been investigated so far. This study addressed three important aspects in this area: effect of IAA on fungal growth and hyphal ramification, IAA biosynthesis in *T. vaccinum* and the controversial effect of IAA on differentiating ectomycorrhizal features. The results of these studies were interpreted as to their relevance in relation to ectomycorrhiza formation and maintanance. This is, to the best of our knowledge, the first study that has comprehensively integrated the aforementioned aspects in trying to break the impasse about our understanding of IAA involvement in ectomycorrhiza.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Organisms and plasmids

Escherichia coli and fungal strains used in this study, together with their description, are summarized in Table 1 and 2 while plasmids are in Table 3. The only plant used for mycorrhiza synthesis was Norway spruce (*Picea abies* (L.) Karsten). Spruce seeds were received from "Thüringer Forstamt Schmalkalden". *Agrobacterium tumefaciens* strain AGL-1, kindly provided by Prof. Marjatta Raudaskoski, University of Helsinki, Finland, was used for *A. tumefaciens*-mediated fungal transformation.

Table 1. *Escherichia coli* strains used

Strain	Genotype	Reference
DH5α	*endA*1, *hsdR*17, *supE*44, *thi*-1, *recA*1, *gyrA*96, *relA*1, Φ80d *lacZ* M15, F^-	GibcoLife Technologies, Karlsruhe, Germany
ArcticExpress	F^-, *ompT*, *hsdS*(r^-_B m^-_B), *dcm*$^+$, Tef^r, *gal*, *end*A, Hte [*cpn*10 *cpn*60 $Gent^r$]	Stratagene, Waldbronn, Germany
BL21-AI	F^-, *ompT*, *hsdS*$_B$ (r^-_B m^-_B), *gal*, *dcm*, *araB*:*T7RNAP-tetA*	Invitrogen, Karlsruhe, Germany

Table 2. *Tricholoma vaccinum* strains used

Strain	Description	Reference
GK6514	Wild type strain isolated from Norway spruce	Kindly provided by G. Kost (University of Marburg)
Tvaldh1-eGFP$^-$1	Derived from GK6514 by overexpressing *ald1*	This study
Tvaldh1-eGFP$^-$2	Derived from GK6514 by overexpressing *ald1*	This study
Tvaldh1-eGFP$^-$3	Derived from GK6514 by overexpressing *ald1*	This study
Tvaldh1-eGFP$^+$1	Derived from GK6514 by overexpressing *ald1* fused to *egfp*	This study
Tvaldh1-eGFP$^+$2	Derived from GK6514 by overexpressing *ald1* fused to *egfp*	This study
Tvaldh1-eGFP$^+$3	Derived from GK6514 by overexpressing *ald1* fused to *egfp*	This study
Tvaldh1-eGFP$^+$10	Derived from GK6514 by overexpressing *ald1* fused to *egfp*	This study

Table 3. Plasmids used

Designation	Genotype/Description	Reference
pDrive	Cloning vector, Amp^R, Kan^R	Qiagen, Hilden, Germany
pBGgHg	Amp^R, Hyg^R, containing the *Agaricus bisporus gpd* promoter	Chen *et al*. (2000)
pNEB193	Amp^R	New England Biolabs, Schwalbach, Germany
pET101/D-TOPO	Amp^R, directional cloning vector for gene expression in *E. coli*	Invitrogen, Groningen, The Netherlands
pNGaldh1	Amp^R, pNEB193::*ald1* under P*gpd* promoter from *A. bisporus*	This study
pNGaldh1S	Amp^R, pNEB193::*ald1*, P*gpd* from *A. bisporus* and 35S terminator from CaMV	This study
pNGaldh1SeGFP	Amp^R, pNEB193::*ald1-egfp* fusion, P*gpd* from *A. bisporus* and 35S terminator from CaMV	This study
pBGaldh1	Amp^R, Hyg^R, pBGgHg::*ald1*, P*gpd* from *A. bisporus* and 35S terminator from CaMV	This study
pBGaldh1SeGFP	Amp^R, Hyg^R, pBGgHg::*ald1-egfp* fusion, P*gpd* from *A. bisporus* and 35S terminator from CaMV	This study

2.1.2. Chemicals, enzymes and antibiotics

All chemicals, reagents, enzymes, and antibiotics were obtained from the following companies: Oligonucleotides were synthesized at MWG Biotech (Ebersberg, Germany); restriction endonucleases as well as other DNA-modification enzymes and purification kits were obtained from Jena Bioscience GmbH (Jena, Germany), New England Biolabs (Schwalbach, Germany), PeQlab (Erlangen, Germany), Gibco Life Technologies (Karlsruhe, Germany), GE Healthcare (Freiburg, Germany), Qiagen (Hilden, Germany) and Invitrogen (Groningen, The Netherlands); chemicals and antibiotics were obtained from Sigma Aldrich (Steinheim, Germany), Fluka (Steinheim, Germany), Serva (Heidelberg, Germany), Merck (Damstadt, Germany), Ferak Laborat GmbH (Berlin, Germany), Miltenyi Biotech GmbH (Germany) and Carl Roth GmbH (Karlsruhe, Germany).

2.1.3. Solutions, reagents and buffers

The composition, and where necessary the pH, of solutions, reagents and buffers used in different experiments are summarized in this chapter.

Microscopy: PME buffer (50 mM PIPES pH 6.7; 25 mM EGTA pH 8.0; 5 mM $MgSO_4$), fixation solution (PME buffer; 4 vol.-% formaldehyde) and embedding medium (0.1M Tris/HCl pH 8.0; 50 vol.-% glycerol; 0.1µg/ml DAPI).

Nucleic acid isolation: DNA extraction buffer (200 mM Tris/HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5 vol.-% SDS), CTAB-NaCl solution (10 vol.-% CTAB; 0.7 M NaCl), DEPC (100 mM Tris/HCl pH 8.0; 10 mM EDTA; 2 vol.-% SDS).

Plasmid preparation: Solution A (50 mM glucose; 25 mM Tris/HCl pH 8.0; 10 mM EDTA), solution B (0.2 M EDTA; 1 vol.-% SDS), solution C (7 M NH_4 acetate).

Nucleic acid analysis: Marker for gel electrophoresis (0.2 vol.-% bromophenol blue; 0.2 vol.-% xylene cyanol; 0.2 vol.-% orange G; 50% sucrose; 1 mM EDTA), 20x TAE electrophoresis buffer (0.8 M Tris/Acetat pH 8,0; 0.02 M EDTA) and ethidium bromide solution (1µg/ml deionised water) [Sambrook *et al*., 1989].

Buffers and solutions for DNA-DNA hybridization: Denaturation solution (0.5 M NaOH; 1.5 M NaCl), 20x SSC (3 M NaCl; 0.3 M Na_3Citrate pH 7-8), standard hybridization buffer (5×SSC; 0.02 vol.-% SDS; 0.1 vol.-% N-Lauroylsarcosin; 1 % blocking reagent(Roche Diagnostics, Mannheim, Germany)), 2×washing buffer (2×SSC; 0.1 vol.-% SDS), neutralisation solution (0.5 M Tris/HCl pH 7.5; 3 M NaCl), 0.5x washing buffer (0.5×SSC; 0.1 vol.-% SDS), detection buffer (10 mM Tris/HCl pH 9.5; 10 mM NaCl), detection washing buffer (0.1 M Maleic acid pH 7.5; 0.15 M NaCl; 0.3 Vol.-% Tween 20), and blocking solution (0.1 M Maleic acid pH 7.5; 0.15 M NaCl; 1 % blocking reagent).

Reagents and buffers for IAA experiments: Salkowski's reagent (Gordon and Weber, 1951): 150 ml concentrated H_2SO_4; 250 ml deionised water; 7.5 ml of 0.5 M $FeCl_3.6H_2O$.

Buffers and solutions for proteomics experiments: Phosphate buffer (0.1 M $Na_4P_2O_7 10H_2O$), separating buffer (1.5 M Tris/HCl pH 8.9); 10 vol.-% SDS; 10 vol.-% APS, stacking/gathering buffer (0.5 M Tris/HCl pH 6.8), sample buffer (62.5 mM Tris/HCl pH 8.6; 10 vol.-% Glycine; 4 vol.-% SDS; 0.005 vol.-% Bromophenol blue), running buffer (25 mM Tris; 192 mM Glycine; 0.1 vol.-% SDS), binding buffer (20 mM Na_2HPO_4; 20 mM NaH_2PO_4; 0.5 M NaCl; 30 mM imidazole), elution buffer (20 mM Na_2HPO_4; 20 mM NaH_2PO_4; 0.5 M NaCl; 0.5 M imidazole), PBS (5.84 g/l NaCl; 14.24 g/l Na_2PO_4; 2.4 g/l NaH_2PO_4).

2.1.4. Media

All media constituents are reported per 1 litre of deionised water. Media were sterilized by autoclaving at 121ºC and a pressure of 1 bar for 30 min. The chemicals which could not be autoclaved, including antibiotics, were filter-sterilized by using 0.22 µm filters (Carl Roth

GmbH, Karlsruhe, Germany), and added to pre-cooled media. Agar was only used for solid media. The following media were used:

Bacterial media: LB-medium (Sambrook *et al*., 1989): 10 g tryptone; 5 g yeast extract; 10 NaCl; 18 g Agar, SOC-medium (Sambrook *et al*., 1989): 20 g tryptone; 5 g yeast extract; 0.5 g NaCl; 10 ml of 250 mM KCl - medium sterilized by autoclaving, cooled and 5 ml and 20 ml of a sterile solutions of 2 M $MgCl_2$ and 1 M glucose respectively added, *A. tumefaciens* MM-medium (10.5 g K_2HPO_4; 4.5 g KH_2PO_4; 1 g $(NH_4)_2SO_4$; 0.5 $C_6H_5O_7Na_32H_2O$; 0.2 $MgSO_47H_2O$; 1 mg thiamine hydrochloride; 2 g glucose), and SB-medium (tryptone 32 g; yeast extract 20 g; NaCl 5 g pH 7.5).

***T. vaccinum* medium:** MMNb (Kottke *et al*., 1987): 0.05 g $CaC1_22\ H_2O$; 0.025 g NaCl; 0.5 g KH_2PO_4; 0.25 g $(NH_4)_2\ HPO_4$; 1 µg $FeC1_36H_2O$; 83 µl 1.2 mg/ml thiamine hydrochloride; 0.15 g $MgSO_4$; 10 g glucose; 20 g malt extract; 1 g peptone from casein; 10 ml trace element solution by Fortin; 20 g agar. Trace element solution (Fortin and Piche, 1979) contained: 3.728 g KCl; 1.546 g H_3BO_3; 0.845 g $MgSO_47H_2O$; 0.575 g $ZnSO_47\ H_2O$; 0.125 g $CuSO_45\ H_2O$.

Spruce seed germination medium (Chilvers *et al*., 1986): 2 g PIPES; 0.1 g KH_2PO_4; 0.2 g NH_4NO_3; 0.1 g $MgSO_47H_2O$; 0.1 g $CaC1_22\ H_2O$; 1 µg $FeC1_36H_2O$; 10 ml trace element solution by Fortin; 10 g Agar. Trace element solution (Fortin and Piche, 1979) was formulated as for MMNb medium.

Mycorrhiza culture medium: MMNa: Same as the afforementioned MMNb medium for *T. vaccinum* but without glucose and malt extract.

2.1.5. Oligonucleotides

For PCR, oligonucleotide primers listed in Table 4 were used.

Table 4. Oligonucleotide primers used

Designation	5'-3' sequence	Origin of primers
Sequencing		JenaGen, Germany; Sambrook *et al*. (1989)
M13 forward	GTAAAACGACGGCCAGT	
M13 reverse	CAGGAAACAGCTATGAC	
cDNA quality check		
rf13a	GCAAGAAAGGCATACAAAACT	This study
rf13b	GCGTCGCTGGTGAAAAT	This study
ald1 fragment		
AD1	(AGCT)GTCGA(GC)(AT)GA(AGCT)A(AT)GAA	This study
AD3	GT(AGCT)CGA(GC)(AT)CA(AGCT)A(AT)GTT	This study
AD6	(GC)CAC(AGCT)TC(GC)T(AGCT)GT(AGCT)TCT	This study
T1	GTCTGCCCATCCAGCATAGT	This study

T2	CAGACAGCCACTAACCGACA	This study
T1b	ACAACCTTGCCGGTAGCTTA	This study
T2b	GCACCTGGATTGACAACACT	This study
T3b	GAACGCATCTTGAACGCATC	This study
T13b	TCAACTGGAGGCCGGTACT	This study
T23b	TCGGAGGGTACAAACAGTCA	This study
T33b	GGTCGTGAGCTAGGCCAGTA	This study
ald1 gene		
aldh1-F1	ATGCCTGGGACTTTCACTCG	This study
aldh1-R1	TCAACAGGAGCAACCCAAGG	This study
ald1 poly(A)		
Poly1	TGGTGCATGGAGTCAGAGAG	This study
dT1	GCCACGCGTCGACTAATACT (dT_{18})(AGC)	This study
ald1-gpd fusion		
gpdF	GAGCTCAGCTTTAAGAGGTCCGCAAG	This study
gpdR	GTGGCGAGTGAAAGTCCCAGGCATGGTACCG CGATAAGCTTG	This study
ORFF	CACAACAAGCTTATCGCCGGTACCATGCCTG GGACTTTCACTCG	This study
ORFR	GGCGCGCCTCAACAGGAGCAACCCAAG	This study
egfp gene & 35S (T)		
EgfpFII	GGCGCGCCATGGTGAGCAAGGGCGAGGAGC TGTTCA	This study
EgfpRII	GTTTAAACGGGATGTGCTGCAAGGCGATTAA GTTGG	This study
35SF	GGCGCGCCTCGACAAGCTCGAGTTTCTC	This study
ald1 expression		
aldh1Ec-F	CACCATGCCTGGGACTTTCACTC	This study
aldh1Ec-R	GAGCTTCATGCCGATGTTTAC	This study
aldRTPCRF	GAAAGCTCTTGGAGCAGGTG	This study
aldRTPCRR	TGGACTGTAGCACCCTCCTT	This study
hgh gene		Hanif *et al*.
Hph-F	AAGCCTGAACTCACCGCGAC	(2002)
Hph-R	CTATTCCTTTGCCCTCGGAC	
egfp gene		Hanif *et al*.
Egfp-1	ATGGTGAGCAAGGGC	(2002)
Egfp-2	TTACTTGTACAGCTCGTC	

2.2. Methods

2.2.1. Culture conditions for different organisms

Bacterial cultures: *E. coli* cultures were routinely grown overnight at 37ºC shaking in LB-medium at 200 rpm. The cells that were specially used for heterologous gene expression studies were grown at 18 ºC to facilitate induction of gene transcription. Transformed *E. coli* cells were allowed to recover in SOC-medium for 1 hr shaking at 37ºC before plating on solid

LB-medium in Petri dishes containing 40 µg/ml X-Gal, for blue-white screening, and overnight incubation of plates at 37ºC.

A. tumefaciens cultures were grown at 28 ºC shaking in liquid LB-medium or standard 'Nutrient broth I' at 200 rpm for 24 hrs while plate cultures were incubated for about 2 days. Where appropriate, media were supplemented with antibiotics for selection of resistant colonies.

***T. vaccinum* fungal cultures:** The fungus was cultured, for various purposes, in MMNb-medium, either liquid (50 ml in 100 ml flask) or solid (Petri dishes). Typically, fungal cultures were established by inoculating fresh medium with 3 fungal mycelial plugs of about 0.4 cm^2, and incubated at RT for about 4 weeks. For DNA or RNA extraction and microscopic analysis of hyphae, the fungus was strictly grown in liquid medium. To study the induction effects of different alcohols and aldehydes on *T. vaccinum ald1* gene expression, liquid MMNb-medium was supplemented with various concentrations of these substrates.

Germination of spruce seeds: Seeds were incubated in de-ionized water over night at RT to facilitate germination. After surface-sterilization by shaking in 30% H_2O_2 for 1-2 hrs, the seeds were rinsed in sterile de-ionized water 3 times under sterile conditions. The seeds were then germinated on solid germination medium (section 2.1.5) in 500 ml Erlenmeyer flask. Germination of seeds was allowed to proceed in a climate chamber KBWF 240 (WTB binder Labortechnik GmbH, Tuttlingen, Germany) with the following conditions: 12 hr day-night regime with an alternating temperature of 23 /17 °C and 80 % relative humidity.

Mycorrhiza cultures [Asiimwe *et al*., 2010; Chilvers *et al*., 1986]: An axenic Petri dish system of synthesizing ectomycorrhiza was used (Chilvers *et al*., 1986). Mycorrhiza was synthesized using solid MMNa-medium both on small (94 mm x 16 mm) and big (135 mm x 20 mm) Petri dishes. For the small Petri dish, spruce seedling shoot was left outside the plate through a hole made in the rim of the Petri dish, while the whole seedling was sealed inside the big Petri dish. The MMNa-medium, which was used to investigate the effect of IAA on mycorrhiza development, was supplemented with 100 µM IAA and 10 µM of its inhibitor (TIBA), either alone or simultaneously added. In some cases, the possibility of enhancement of ectomycorrhiza formation by glucose was tested by amending MMNa-medium with 2 g/l glucose. Mycorrhiza was synthesized as follows: Cellophane membranes (Wilhelm Isermann KG, Walsrode, Germany) were cut, in a circular form, shortly boiled to remove production residues and rinsed in de-ionized water. The membranes, separated by filter papers (90 mm diameter), were sealed in aluminium foil and sterilized by autoclaving at 121ºC and a pressure of 1 bar for 30 min. Under sterile conditions, a seedling was horizontally placed on MMNa

plate, sandwiched between two sterile membranes to avoid direct contact with the medium, and the plate was then sealed with parafilm. To avoid direct root exposure to sun light, aluminium foil was used to shade the petri-dish, after which the seedlings were incubated in a growth chamber (12 hr day-night regime with an alternating temperature of 23 /17 °C and 80 % relative humidity). After the seedlings had differentiated the first lateral roots (2-4 weeks after seedling transfer from germination medium to MMNa-medium), they were inoculated with 4 mycelial plugs of about 0.4 cm^2, which were placed near roots. At regular intervals, the seedling roots were visually and microscopically checked for development of mycorrhizal features.

2.2.2. Analysis of IAA production by *T. vaccinum*

The production of IAA by *T. vaccinum* was investigated by culturing the fungus in liquid MMNb-medium supplemented with various concentrations of IAA precursors indole-3-acetaldehyde and tryptophan. Also, the possible effect of D'orenone (kindly provided by Dr. Doreen Schchtschabel, Jena, Germany), an auxin polar transport inhibitor (Schlicht *et al*., 2008), on production of IAA by *T. vaccinum* was tested by exogenous application of different concentrations of the compound to the fungal cultures. Cultures were incubated in dark at RT for about 4 weeks before determining IAA concentration in culture filtrates. To test the transient fungal IAA production over time, culture filtrates were sampled every week beginning at the time of culture establishment. Fungal IAA production was assessed by determining IAA quantities in culture filtrates using the following methods:

Calorimetric method: Salkowski assay (Godon and Weber, 1951) was used to assess IAA production in fungal culture filtrates. The assay was performed by vigorously mixing 250 µl of fungal culture filtrates, obtained after centrifugation at 13000 rpm for 20 min, with 1 ml of Salkowski's reagent (section 2.1.4), and incubating at RT for 20 min before measuring absorbance at 535 nm. Quantification of IAA in the culture filtrates was based on the comparison with IAA standard curve developed using pure synthetic IAA.

Gas Chromatography-Mass Spectrometry (GC-MS): GC-MS was used to validate the IAA production by *T. vaccinum*. IAA was extracted and quantified according to a protocol from Dimkpa *et al.* (2008) with a few modifications: 5 ml of fungal culture filtrates, obtained from fungal liquid cultures, either supplemented or not, with different concentrations of indole-3-acetaldehyde and tryptophan as IAA precursors, instead of 3 ml, were used as starting material for IAA extraction. Also, a different calibration curve, for quantification of

IAA, was developed by adding known amounts of IAA to 5 ml of pure fungal medium (MMNb) followed by IAA extraction and quantification as done in Dimkpa *et al.* (2008).

2.2.3. Quantification of IAA effects on *T. vaccinum* and mycorrhiza

Effect on fungal biology: The effect of IAA both on radial fungal growth and hyphal branching was studied. The experiments were set up as in section 2.2.2, with a few modifications. To study the IAA effect on fungal hyphal branching, the liquid MMNb medium was supplemented with indole-3-acetaldehyde (0.05 mM, 0.1 mM and 0.2 mM), the IAA polar auxin transport inhibitors D'orenone (800 nM and 4 µM) and TIBA (10 µM), tryptophan (0.5 mM and 2.5 mM) and synthetic IAA (100 µM and 500 µM). IAA was either added alone or simultenously with its inhibitor TIBA. On top of a control, ethanol up to a final concentration of what was used to dissolve synthetic IAA and TIBA or D'orenone (0.015%) was used as a blank. The effect of these treatments on fungal hyphal branching was microscopically studied by observing the extent and pattern of the branching.

For experiments involving investigation of IAA effect on radial fungal growth, solid MMNb-medium amended with synthetic IAA (100 µM), tryptophan (500 µM) and TIBA (10 µM), either applied alone or simultenously with IAA or tryptophan, were used. A blank, as described above, was used. Fungal mycelial radial growth was quantified, in real time, by measuring the diameter of the fungal colonies. The measurements were commenced about 1 week, when aerial mycelium had started forming, and stopped 4 weeks after culture establishment.

Effect on mycorrhiza development: Solid MMNa-medium with or without glucose (2 g/l), and amended with synthetic IAA (100 µM) and TIBA (10 µM), singly or simultenously added, were used. A blank, as described above, was used. The co-cultivation of spruce seedlings and *T. vaccinum* was done on big plates (135 mm x 20 mm), with the whole seedling sealed inside the plate to minimize contaminations (Asiimwe *et al*., 2010). The effect of IAA on mycorrhiza development was quantified by regular microscopic observations for ectomycorrhizal features notably the fungal mantle and Hartig' net. Plant growth and development parameters were also quantified in the mycorrhiza cultures by counting the number of newly formed lateral roots and leaves/needles on spruce seedlings.

2.2.4. Molecular biology methods

2.2.4.1 Nucleic acids preparations

Genomic DNA isolation: Genomic DNA of *T. vaccinum* was isolated by using CTAB/NaCl-extraction method (Rozman and Komel, 1994; Wendland *et al*., 1996) with a few modifications. The fungal mycelium, from liquid culture, for DNA extraction was prepared by rinsing with de-ionized water using a nylon mesh, dried and frozen at -20 ºC before the commencement of the DNA extraction procedure. For DNA extraction, the mycelium was ground, using a mortar, pestle and liquid nitrogen, to fine powder. About 50 mg mycelial powder were weighed in a 1.5 ml Eppendorf tube and used for DNA extraction as follows: 600 µl DEP and 80 µl pre-warmed CTAB/NaCl solution were added after which NaCl concentration was adjusted to 0.7 M by adding about 95 µl 5 M NaCl. The mixture was then incubated for about 60 min at 65 °C, cooled at RT for about 10 min, and centrifuged for 10 min at 13000 rpm. The supernatant was subjected to chloroform extraction by mixing it with 600 µl chloroform and incubating the mixture on ice for 15 min interrupted by regular mixing before centrifuging (10 min, 13000 rpm, 4 °C). Using the supernatant, the whole procedure of CTAB/NaCl solution/Chloroform extraction was repeated twice. DNA was precipitated by adding 0.7 volume isopropanol to the new supernatant, incubating on ice for 30 min followed by centrifugation (25 min, 13000 rpm, 4 °C). The DNA pellet was then washed with about 500 µl 70 vol.-% ethanol by centrifugation (3 min, 13000 rpm, 4 °C). After speed vacuum-drying the pellet for 2-3 min, it was re-suspended in about 50 µl either sterile de-ionized water or elution buffers from different companies, depending on the intended use of DNA, and incubated in water bath at 70°C for 10 min to facilitate DNA suspension and denaturing of DNases.

Isolation of DNA fragments from agarose gels: Agarose gel electrophoresis (Sambrook *et al*., 1989) was done on all nucleic acid samples to ascertain their purity. The nucleic acids were run on an agarose gel (0.8 – 3.5 %), prepared in 1 % TAE buffer, and visualized under UV light after staining in ethidium bromide. Where necessary, DNA fragments were re-isolated from the gels by using a gel extraction kit supplied by Qiagen (Hilden, Germany). However, in complicated cloning procedures involving large inserts and vectors, a different strategy, aimed at improving the quality of DNA, was used. In this strategy, agarose gels were neither stained with ethidium bromide nor exposed to UV light, but band positions determined by reference stained gels, after documentation by a ruler. DNA from such gels was then re-isolated, for subsequent ligations, as follows: Agarose gel pieces were homogenized in a 1.5

ml Eppendorf tube with a pestle to fine particles. 500 µl de-ionized water and 500 µl "Roti-phenol for RNA/DNA isolation" (Carl Roth GmbH, Karlsruhe, Germany) were added, after which the mixture was vortexed and frozen in liquid nitrogen for about 10 min. The mixture was then centrifuged (10 min, 13000 rpm, RT). The aqueous phase was washed with chloroform before precipitating DNA with a mixture of 0.1 and 0.7 volume sodium acetate (3 M) and isopropanol respectively.

Plasmid DNA isolation: For isolation of high quality bacterial plasmid DNA to be used for sensitive applications like sequencing, 'PeqGOLD plasmid miniprep kit I' supplied by PeQlab (Erlangen, Germany) was used. Otherwise, the routine plasmid isolation was done using the alkaline lysis method (Sambrook *et al*., 1989) as follows: About 1.5 ml of bacterial overnight culture was centrifuged (5 min, 13000 rpm, RT) in an Eppendorf tube. The pellet was re-suspended in 150 µl solution A by vortexing, and incubated at RT for 5 min to destabilize the bacterial cell wall. 400 µl solution B were added to the mixture and incubated at RT for 15 min for further lysis of cell walls. 300 µl solution C were added, and the mixture was incubated on ice for 15 min before centrifugation (30 min, 13000 rpm, RT). The plasmid DNA in the supernatant was precipitated by incubating with 600 µl isopropanol for 10 min before centrifugation (20 min, 13000 rpm, RT). The plasmid DNA pellet was then washed with about 500 µl 70 vol.-% ethanol by centrifugation (3 min, 13000 rpm, 4 °C). After 2-3 min speed vacuum-drying, the pellet was re-suspended in about 20 µl sterile de-ionized water, and incubated in water bath at 70°C for 10 min to facilitate DNA suspension denaturation of DNases.

RNA isolation: Total RNA was isolated from *T. vaccinum* by using RNeasy plant mini kit from Qiagen (Hilden, Germany). Isolated RNA was stored, in aliquots, at -80°C until use. Before use, RNA was purified by removing the residual DNA from the solution using a 'DNAfree-Kit' from Ambion (Austin, Texas, USA).

2.2.4.2 DNA-DNA hybridization by Southern blotting

Southern blotting was carried out on *T. vaccinum* genomic DNA to detect *ald1* sequence in the genome of the wild type strain. However, this procedure was also carried out to determine the nature of integration of the hygromycin resistance transgene (*hgh*) in the gDNA of transformants derived from *A. tumefaciens*-mediated transformation (ATMT) of *T. vaccinum*. The procedure was done according to the protocol 'DIG system for filterhybridisation' (Roche Diagnostics, Mannheim, Germany), based on the DIG-dUTP insertion in a nucleic acid fragment as a probe, followed by DIG-antibody binding and chemiluminescent detection

in a specific manner. To prepare DNA probes, DNA fragments, which were earlier isolated from agarose gels, were labeled with DIG-dUTP as follows: Fragment DNA was first denatured (10 min 95°C) and cooled on ice. 2 µl each of hexanucleotide and 'dNTP labeling' mixtures and 1 µl Klenow-Enzyme (stock solution 0.1 U/µg) were added. The mixture was incubated for 20 hrs at 37°C before stopping the labeling with 2 µl EDTA (stock solution 200 mM).

DNA-DNA hybridization was performed as follows: Genomic DNA was first digested overnight with restriction enzymes (either single or double digestion). Then, the restriction mixture was run on a 0.8% agarose gel and documented with a ruler under UV light after ethidium bromide staining. DNA in the gel was depurinated by shaking the gel in 250 mM HCl for 10 min at RT. DNA was denatured by rinsing the gel twice for 15 min in denaturation solution followed by short rinsing in de-ionized water to get rid of excess denaturation buffer. The gel was then rinsed twice for 15 min in neutralization solution I before it was transferred to a nylon membrane (Macherey-Nagel, Düren, Germany) for overnight blotting by capillary transfer. The nylon membrane, now containing DNA, was incubated for 30 min in an oven (120 °C) to fix the DNA onto the membrane. Then, the membrane was transferred to a hybridization tube containing standard hybridization buffer and incubated rolling in hybridization oven (68 °C) for 2-3 hrs to bind unspecific sites on the membrane. The standard hybridization buffer was removed and replaced with a fresh one containing the DIG-labeled probe, which was first denatured (10 min 95 °C, cooling on ice). Hybridization was allowed to proceed overnight at 68 °C by rolling of the tube in the hybridization oven. The membrane was then washed twice for 5 min in 2×washing buffer at RT, followed by two times washing for 15 min in 0.5×washing buffer at 68 °C. The membrane was equilibrated in detection washing buffer for 1 min and cooled to RT. It was then incubated in blocking solution at RT for 30 min to bind more unspecific sites followed by incubation with another fresh blocking solution containing 1:10000 alkaline phosphotase-coupled anti-DIG antibody for 30 min at RT. The membrane was washed twice for 15 min in fresh detection washing buffer at RT followed by 2 min equilibration in detection buffer. The membrane was then transferred to a cling film and 1-5 drops CPD-Star solution (Roche Diagnostics, Mannheim, Germany) applied before wrapping the whole membrane in the cling film and incubated for 5 min at RT. After this, the membrane was wrapped in a fresh cling film, fixed on a board and covered by X-ray film (X-OMAT AR, Kodak, New York, USA) in a dark room. The board was then incubated at 20°C-37°C for 1 min – 2 hrs, depending on the intensity of the signal, before the detection of chemiluminescence on X-ray film.

2.2.4.3 Polymerase chain reaction (PCR)

General routine PCR: For routine amplification of DNA fragments, either for cloning, sequencing or detection of specific fragments, a general gene specific PCR procedure, outlined below, was performed. The PCR reaction mixture typically contained 2 µl PCR buffer (10x), 2 µl dNTPs (2 mM), 2 µl each forward and reverse primers (5 pmol/µl), 0.1 µl Taq DNA polymerase (5 U/µl), 4 µl enhancer (5x) and 1 µl DNA sample (40 – 100 ng/µl) in a final volume of 20 µl. As a quick identification of positive clones in heterologous expression of *ald1* gene in *E. coli*, a single colony was used as a template, instead of DNA. Where high fidelity of a DNA polymerase was required, a proofreading polymerase (Pfu-x) was used. Thermocycling was carried using a 'Thermalcycler' (Biometra, Göttingen, Germany) as follows: Initial DNA denaturation (94°C, 2 min), denaturation (94°C, 30 sec), annealing (temperature chosen to suit the oligonucleotide primer pair, 30 sec), initial elongation (72°C, time chosen to suit expected size of amplification product) and final elongation (72°C, time dependent on the downstream use of amplification product). A total of 30 – 34 cycles were carried out.

Reverse Transcriptase-PCR (RT-PCR): RT-PCR was carried out to synthesize cDNA from mRNA of *T. vaccinum*. The cDNA was synthesized by using the 'iScriptTM cDNA Synthesis Kit' supplied by Bio-Rad laboratories (Hercules, CA, USA). 500 ng to 1 µg of total RNA was used as the template in final reaction volume of 20 µl consisting of other reaction components, as recommended by the manufacturer. One PCR cycle [(25°C, 5 min), (42°C, 30 min), (85°C, 5 min)] was carried out.

Real time Reverse Transcriptase PCR (Real time RT-PCR): The transcription of *ald1*, as induced by ethanol and various aldehydes, was analyzed with the help of real time RT-PCR. *T. vaccinum* for this study was cultured in liquid MMNb-medium supplemented with 0.01% and 0.1% ethanol, 0.01 mM and 0.1 mM indole-3-acetaldehyde, and 0.1 mM benzaldehyde. Mycelium was then harvested, and total RNA extracted before cDNA synthesis from the mRNA fraction. 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, according to the manufacturer recommendations. To increase the accuracy of the PCR procedure, replicates were included at all crucial levels of the procedure i.e. biological, RNA and cDNA replicates. Thermalcycling was carried out using a 'Smart Cycler' supplied by Cepheid (Sunnyvale, CA, USA). The *ald1* mRNA accumulation was quantified in absolute terms (Bustin, 2000) by comparing transcript levels to a standard curve generated by using *ald1* cDNA cloned in pDrive. Transcription was, therefore, monitored in real time by amplification of a 132 bp *ald1*

fragment using aldRTPCRF and aldRTPCRR primers. The amplified *ald1* sequence region spans an intron, an attribute that helped to control amplification target artifacts against gDNA contamination.

Thermal Asymmetric Interlaced (TAIL)-PCR: In order to isolate unknown sequences flanking *ald1* fragment, earlier identified using RNA fingerprinting (Krause and Kothe, 2006), which was aimed at cloning the full length gene, TAIL-PCR was carried out. This PCR, which utilizes nested sequence-specific primers together with an arbitrary degenerated primer, was carried out according to Liu and Whittier (1995) and Liu *et al.* (1995) with modifications. Thus, gene-specific and arbitrary degenerated primer pairs were carefully chosen to facilitate isolation of both the 5' and 3' previously unknown sequences of the *ald1* fragment. Thermocycling was carried out using a 'Thermalcycler' (Biometra, Göttingen, Germany). Different TAIL-PCR reactions, consisting of three rounds of PCR with three gene-specific primers and an arbitrary degenerate primer (AD), were carried out as follows:

A typical first round TAIL-PCR reaction contained 2 µl PCR buffer (10x), 0.5 µl dNTPs (2 mM), 1.6 µl gene-specific primer (5 pmol/µl), 8.0 µl AD1, AD2 or AD3 primer (10 pmol/µl), 0.5 µl Taq DNA polymerase (5 U/µl), 4 µl enhancer (5x) and 1 µl gDNA (40 ng/µl) in a final volume of 20 µl. The first round PCR cycling conditions were [92°C (2 min), 95°C (1 min), 94°C (30 sec), 59°C (1 min), 72°C (2 min) with 5 cycles]; [94°C (30 sec), 25°C (3 min) followed by ramping at a rate of 4°C/sec to 72°C over 3 min, 27°C (2 min)]; [94°C (30 sec), 59°C (1 min), 72°C (2 min), 94°C (30 sec), 59°C (1 min), 72°C (2 min), 94°C (30 sec), 44°C (1 min), 72°C (2 min) for 15 cycles] and a final elongation of 72°C (5 min).

The second round PCR reaction contained the same reaction mixture except the AD primer and the template. The template used was 1 µl of the 50x diluted PCR products from the first round of PCR, and AD primer was scaled down to 4 µl (10 pmol/µl) to decrease unspecific amplification. Thermocycling for the second round of PCR was 94°C (30 sec), 59°C (1 min), 72°C (2 min), 94°C (30 sec), 59°C (1 min), 72°C (2 min), 94°C (30 sec), 44°C (1 min), 72°C (2 min) for 12 cycles and a final elongation of 72°C (30 min).

In the third round of PCR, the reaction volume was scaled up to 100 µl. 1 µl of 10x diluted second round PCR products was used, representing a final 1000x dilution of the second round PCR products, to further minimize unspecific amplification. Thermocycling conditions for this PCR were 94°C (2 min), 44°C (1 min), 72°C (2 min) with 20 cycles and a final elongation of 20 min.

The consecutive first, second and third round PCR products were run alongside each other on an agarose gel to differentiate specific products from non-specific ones, based on the size differences of bands as compared to gene-specific primer positions in the *ald1* fragment.

Poly(A) region PCR: A special PCR procedure was carried out to isolate the 3' flanking sequences of *ald1*, including the polyadenylation (Poly(A)) signal and tail. This was done using a gene-specific, Poly1, and a modified oligo (dT_{18}) dT1 primer pair. The oligo (dT_{18}) was modified by attaching an adaptor sequence (5'-GCCACGCGTCGACTAATACT-3') to the 5' end and a single nucleotide anchor V (A, G, or C) to the 3' end of oligo (dT_{18}) to stabilize the primer and increase annealing temperature to match the gene-specific primer for better amplification of target. The general PCR procedure, under specific conditions (outlined above), was used for this task.

2.2.4.4 DNA modifications

Restriction: Typically, restriction digests of DNA were carried out in appropriate buffers and according to instructions of the manufacturers using a final volume of 20 µl. Restriction mixtures were incubated overnight at recommended temperatures before analysis by agarose gel electrophoresis. Where necessary, after restriction digest of plasmid DNA, 5' phosphate groups were removed to avoid re-ligation of compatible ends. This was carried out by using Antarctic phosphotase enzyme (New England BioLabs, Schwalbach, Germany) according to the manufacturer recommendations.

Ligation: Ligation of restriction fragments was carried out overnight at 16°C in a final volume of 5 – 20 µl, depending on the nature of the ligation procedure. The molar ratios of vector: insert DNA used was 1:1 – 1:16, with higher ratios being used in complex ligations. Also, in these complex ligations, ligation buffers supplied by companies were supplemented with additional ATP (5 µM) to cater for its possible degradation over a period of time. Except for routine ligations involving cloning vectors, T4 DNA ligase (Jena Bioscience, Jena, Germany) was used to enhance ligation according to the manufacturer recommendations.

2.2.4.5 DNA sequencing

For routine screening of the cloned PCR fragments, the insert DNA was sequenced at JenaGen GmbH (Jena, Germany) while the full length *ald1* gene and other clones, which required a high sequencing accuracy, were sequenced at GATC Biotech (Konstanz, Germany). The standard M13 sequencing primers (table 4) were used, either solely or in

combination with a*ld1* gene-specific primers, depending on the size of the insert to be sequenced. In all the sequencing operations, plasmid DNA of the vectors containing inserts was used. Plasmid DNA for sequencing was prepared by using a "Plasmid Miniprep Kit" (PeQLab, Erlangen, Germany).

2.2.4.6 Sequence analysis

DNA and protein sequences were assembled and analyzed by using DNASTAR or Lasergene (DNASTAR Inc., USA) softwares. Using these softwares, the sequences were aligned by Clustal W method (Thompson *et al*., 1994), embedded in Megalin program. The nucleotide and protein sequence identity were determined with the help of 'BLAST search' (http://www.ncbi.nlm.nih.gov/blast) to access the related sequences in the GenBank database. Putative localization of Ald1 enzyme in a cell was predicted by analyzing the protein sequence with the help of WoLF PSORT software (http://wolfpsort.org). The putative Ald1 protein molecular weight was calculated using the program ExPASy (http://www.expasy.org/tools).

2.2.4.7 Reconstructing phylogeny

The phylogenetic relationship between Ald1 with other fungal aldehyde dehydrogenase enzymes was investigated. Amino acid sequences of related aldehyde dehydrogenase enzymes were retrieved from NCBI GenBank and genomes, using Ald1 protein sequence as a query in "tblastn" and "blastp" searches. The sequences were then 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. Phylogenetic reconstruction was performed under Bayesian inference using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). Amino acid substitution frequencies were assumed to vary over the alignment. Substitution rate variation among sites was taken into account by using four classes of gamma distribution. Two runs, each with 2000000 generations in four chains, were performed, sampling every 100 generations, with a burn-in of 25 percent. Results were evaluated with TRACER v1.4 (Rambaut & 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 (Stamataka, 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 (Rambaut, 2009).

2.2.4.8 Transformation of different organisms

Bacterial transformation: *E. coli* and *A. tumefaciens* were routinely transformed by using electroporation. The exception to the rule was transformation of either *E. coli* with large plasmids or *E.coli* strains (ArcticExpress and BL21-AI) (table 1) used for heterologous *ald1* gene expression, where heat shock was used to increase transformation efficiency. Both the preparation of calcium competent *E. coli* DH5α cells and the subsequent transformation by heat shock were done according to Inoue *et al*. (1990). Transformation of one shot competent cells ArcticExpress and BL21-AI, and subsequent induction of protein production was done according to the suppliers' recommendations.

Electrocompetent *E. coli* cells were prepared as follows: 10 µl of previously prepared electrocompetent *E.coli* DH5α was cultured overnight in 20 ml LB medium at 37°C. 5 ml of the overnight culture were added to freshly prepared 500 ml SB medium, and incubated shaking at 37°C for 3-4 hrs until OD_{600nm} reached 0.6. The bacteria cells were then collected by centrifugation (4000 rpm, 15 min, 4°C) and washed once with sterile water by centrifugation as before. The cells were then washed twice with sterile 10 % glycerol before eluting the pellet in 2 ml 10 % glycerol. 50 µl aliquots were kept frozen at -80°C until use.

Electrocompetent *A. tumefaciens* cells were prepared as follows: A fresh colony of *A. tumefaciens* (AGL-1) was grown in 5 ml LB medium containing 50 µg/ml rifampicin for about 24 hrs shaking (150 rpm, 28°C). 100 ml fresh LB medium containing 50 µg/ml rifampicin were inoculated with 1 ml of the overnight culture, and incubated overnight shaking (150 rpm at 28°C) until OD_{600nm} reached 0.4-0.6. The bacteria cells were then collected by centrifugation (4000 rpm, 10 min, 4°C), washed once with sterile de-ionized water and twice with 10% glycerol by centrifugation as before. Aliquots of 40 µl were kept frozen at -80°C until use.

50 µl electrocompetent *E. coli* cells were transformed with 1-3 µl of ligation mix while 40 µl electrocompetent *A. tumefaciens* cells were transformed with 1 µl plasmid DNA (about 3 ng/ µl). Electrotransformation of the bacterial cells was carried as follows: After thawing the cells on ice, they were gently mixed with DNA samples and transferred to electroporation cuvette. Electroporation was carried out by using 'Gene Pulser II' supplied by Bio-Rad laboratories

(Hercules, CA, USA) at 2.5 kV, 25 µF and low range/high range resistance of 200/800 Ohms. The transformed cells were immediately incubated shaking (150-200 rpm) in 500-900 µl SOC medium for about 1 hr at 28°C and 37°C for *A. tumefaciens* and *E. coli* respectively, in order to recover from transformation shock, before plating.

Fungal transformation: Transformation of *T. vaccinum* with *A. tumefaciens* (AGL-1) carrying plasmids containing *ald1* overexpression cassettes was performed using a recently optimized protocol (Schlunk, personal communication) based on Pardo *et al.* (2002) and Hanif *et al.* (2002) protocols for *A. tumefaciens*-mediated transformation (ATMT) of fungi. *T. vaccinum* to be used for transformation was grown on about 1 cm^2 dialysis Saatifil PES 41/23 W WT nylon membranes (SaatiTech, Veniano, Italy) overlying solid MMNb-medium for 1- 2 weeks until aerial mycelium was formed. The membranes were then inoculated with about 50 µl *A. tumefaciens* culture, which was prepared as follows: *A. tumefaciens* was grown overnight shaking (28°C, 200 rpm) in 4 ml 'Standard Nutrient broth I' (Carl Roth GmbH, Karlsruhe, Germany) containing 50 µg/ml kanamycin. Bacterial cells were collected by centrifugation (4000 rpm, 20 min, 4°C). The pellet was re-suspended in 4 ml induction medium [MM-medium (section 2.1.5) containing 50 µg/ml kanamycin and 200 µM acetosyringone]. The bacterial suspension was then incubated shaking for about 6 hrs (29°C, 200 rpm) until OD_{600nm} of 0.1 was reached. Co-cultivation plates were incubated at RT for 2 days before the membranes were transferred to MMNb-medium selection plates containing 25 µg/ml hygromycin B, 200 µg/ml cefotaxime and 100 µg/ml ampicillin. After about 2 weeks, putative transformants were screened by using PCR methods before transferring them to fresh selection plates, as before, for the second round of selection. After the second selection, transformants were again screened by using PCR and Southern blotting methods.

2.2.5. Protein methods

2.2.5.1 Protein preparation

Crude proteins from *E. coli* cells expressing *ald1* were extracted according to the following procedure: *E. coli* cells were pelleted by centrifugation (4000 rpm, 15 min, 4°C) and re-suspended in 0.1 M sodium pyrophosphate solution. The bacterial cells were then sonicated at 3 cycles for 3 min on ice and centrifuged (12000 rpm, 20 min, 4°C). The crude proteins, in supernatant, were collected and stored frozen at -20°C until use.

2.2.5.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

One dimensional SDS-PAGE was used to analyze proteins extracted from *E. coli* cells expressing *ald1*, under denaturing conditions, by fractionating before visualization after staining. The procedure was carried out as follows: First, protein concentrations were quantified by using the Bradford method (Bradford, 1976). Then, about 100 µg total soluble proteins were loaded on 4.2% stacking/gathering gel (3.6 ml de-ionized water, 0.63 ml gathering buffer, 0.7 ml (Bis)Acrylamide, 50 µl SDS, 5 µl TEMED, 200 µl APS) and fractionated on 11% resolving/separating gel (5.1 ml de-ionized water, 1.25 ml separating buffer, 3.6 ml (Bis)Acrylamide, 100 µl SDS, 20 µl TEMED, 38 µl APS), which was cast immediately beneath the stacking gel. Before loading the gel, proteins were boiled in a loading buffer containing SDS at 95°C for 5 min. The stacking gel and resolving gel were run at 100 V and 180 V respectively at 4°C. The resolving gel was then Coomassie-stained for 2 hrs at RT before de-staining overnight at RT.

2.2.5.3 Western blot analysis

Western blotting was carried out, on total soluble proteins previously separated by SDS-PAGE, to specifically target Ald1 protein, which was His-tagged at the C-terminal end, by using Anti-His antibodies (Miltenyi Biotech GmbH, Germany). First, proteins in SDS-PAGE gel were transferred to nitrocellulose membrane (GE Healthcare, Freiburg, Germany) using the standard blotting method; the protein transfer was carried out at 2.5 mA/cm^2 gel size for 1 hr at 4ºC. Then, immuno-detection was carried out as follows: To reduce background noise, the membrane was blocked by incubating in a blocking solution (3% milk powder) for 1 hr after which it was overlaid with Anti-His antibody (1:7000) for 1-2 hrs on a shaker. This was followed by two washing steps with PBS solution (5 min each) before drying the membrane. The membrane was then incubated with ECL detection reagent (GE Healthcare, UK) for 5 min, exposed to X-ray film for 30 sec before developing the film.

2.2.5.4 Enzyme activity assays

ALDH activity on crude enzyme extracts was carried out according to Guru and Taranath-Shetty (1990) and Isobe *et al.* (2007) with modifications. The 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 30ºC, except acetaldehyde reactions carried out

at 25ºC because of its high volatility, were started by addition of aldehydes, which was done after about 30 sec of pre-reaction. The enzyme activity was measured spectrophotometrically by monitoring the change in absorbance at 340 nm, which is a widely accepted wavelength at which reduction of NAD^+ and $NADP^+$ to NAD(H) or NADP(H) respectively takes place. To eliminate a possible non-enzymatic reaction of NAD^+ or $NADP^+$ with aldehydes to form a complex, which also contributes to increase in absorbance at 340 nm (Guru and Taranath-Shetty, 1990), enzyme activity was measured against a substrate blank (cofactor + aldehyde), as opposed to enzyme blanks (cofactor + enzyme) used by many investigators. Enzyme activity was expressed as change in absorbance per min relative to the control, which was a protein extract from *E. coli* cells not expressing *ald1*. Three replicates for each reaction were used.

2.2.6. Microscopy

2.2.6.1 Specimen preparation

Preparation of root cryosections and fungal mycelium: Mycorrhized spruce seedling roots were cut into about 20 µm thick cyosections using the cryomicrotome Leica CM 1100 (Leica Microsystems GmbH, Nussloch, Germany) at -20°C as follows: About 5 mm long root section was incubated in fixation solution (section 2.1.4) at 4°C for 1-2 days. The root section was then fixed onto the cryomicrotome pre-cooled pistil by applying several layers of tissue freezing medium (Leica Microsystems GmbH, Nussloch, Germany) on the pistil and around the root section, with 5-10 min freezing interval, after which it was incubated freezing for a period of 1 hr to overnight. Cryosections were cut from the root, picked with the help of a glass slide and incubated in fixation solution (section 2.1.4) at 4°C until use.

For studying the effect of IAA on *T. vaccinum* fungal hyphae morphology, the fungus was grown in liquid MMNb-medium supplemented with various IAA and precursor concentrations (section 2.2.3), after which actively growing mycelium in embedding medium (section 2.1.4) was used for microscopy. The mycelium of the *egfp* transformants for eGFP protein visualization, also obtained from liquid cultures, was suspended in sterile de-ionized water before examining the eGFP-associated green fluorescence emission of the detached mycelium on an LSM 510 META confocal laser-scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Staining of specimens: For ordinary fluorescent microscopy, mycorrhized root cryosections or fungal mycelium were transferred to microscopic glass slides (Carl Roth GmbH, Karlsruhe,

Germany), and stained with a 1:800 DAPI solution in embedding medium (section 2.1.4), which stains nucleic acids, by incubation for about 5 min at RT before viewing under microscope.

2.2.6.2 Object viewing

Both binocular and ordinary fluorescent microscopic observations were routinely carried out to identify ectomycorrhizal features. For observations of the effect of IAA on fungal hyphae morphology, only fluorescent microscopy was done. Binocular microscope KL 1500 (Zeiss, Jena, Germany) was used to observe the fungal mantle around a mycorrhized root, as a first line of evidence of mycorrhiza formation. Ordinary fluorescent microscopy was carried out using the microscope 'Axiophot 2' (Zeiss, Jena, Germany) by using filter 02 (Zeiss), with 310-355-400 nm excitation and 405-450-540 nm emission. Microscopic images were obtained with the help of a digital camera, acting as an interface between a microscope and computer, after which the images were processed with 'SPOT advanced' software (Diagnostics instruments, USA).

The expression of Ald1 fused to eGFP was monitored on an LSM 510 META confocal laser-scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

The green fluorescence emission associated with eGFP was detected using an argon laser with 488 nm excitation and 505 nm long pass (LP 505) emission. Images were obtained with a 40x water objective.

2.2.7. Statistical analysis

Statistical analysis was carried out using Origin 7.0 program (OriginLab, USA). Data were analyzed for variance (ANOVA), and where significant, treatments were separated by Tukey test, which is based on studentized range distribution.

3. RESULTS

3.1. Isolation and characterization of *ald1* from *T. vaccinum*

3.1.1. Isolation of *ald1*

For the isolation of full-length *ald1* from the basidiomycete ectomycorrhizal fungus *T. vaccinum*, a 1.986 kb fragment of this gene (Krause, 2005) was used. Subsequently, TAIL-PCR was used to isolate both the 5' and 3', previously unknown, sequences of *ald1*. Using an arbitrary degenerated primer AD3 and gene-specific primers T1 and T2, a 307 bp fragment was amplified at the 5'end of *ald1,* after the second round of PCR (Fig. 7A).

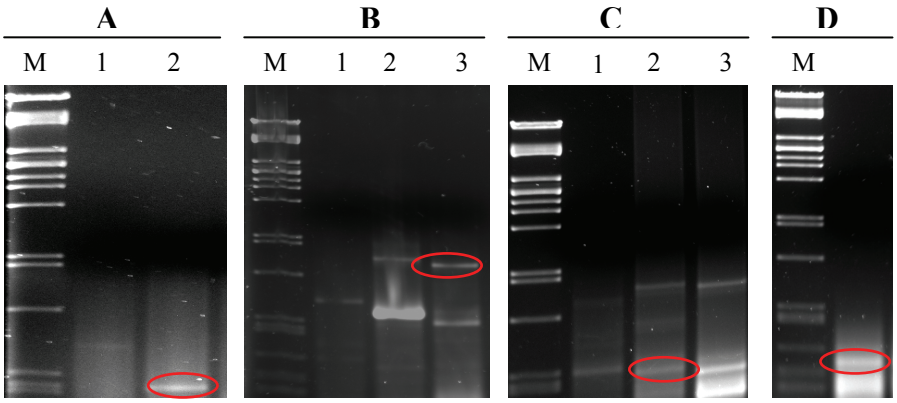


Fig. 7. Agarose gel analysis of PCR products for the isolation *tvaldh1* gene from *T. vaccinum*. TAIL-PCR was used to isolate the 5' flanking sequences (A and B) and 3' flanking sequences (C) while non-coding sequences at the 3' end were isolated by "poly (A) PCR" (D). Lanes denoted 1, 2, and 3 represent the first round, second round and third round of PCR reactions respectively, and M the size marker *λPst*I. The marked fragments represent the correct amplifications.

The next 5' TAIL-PCR with gene-specific primers T1b, T2b and T3b, which were designed in the new sequence, and an arbitrary degenerated primer AD1, yielded an 847 bp new fragment (Fig. 7B) after the third round of PCR. Sequence analysis revealed that the new 847 bp sequence included the *ald1* start and the 5' flanking sequences. Next, an attempt to isolate the unknown 3' sequences using gene-specific primers T13b, T23b and T33b in combination with an arbitrary degenerated primer AD6 yielded a 487 bp fragment (Fig. 7C) after the second round of PCR. Sequence analysis revealed a stop codon in the new sequence. In order to isolate the characteristic 3' flanking sequences, a special PCR, the "Poly (A) region PCR", was used. This PCR yielded a 213 bp fragment (Fig. 7D) of the 3' flanking region, including all the major characteristic sequences.

3.1.2. Sequence analysis of *ald1*

The full genomic sequence of *ald1* and its flanking regions were generated by combining all fragment sequences. Sequence overlap analysis of all fragments showed that the entire gene, which contains an ORF of 2,448 bp, was obtained (Fig. 8). In order to determine the coding sequence, *ald1* cDNA was amplified using primers aldh1-F1 and aldh1-R1. After sequencing, the fragment was compared to the genomic sequence. To further characterize the intron start and end (splicing regions), the GT----AG rule (Breathnach *et al*., 1978) was applied. Furthermore, the translated amino acid sequence of the putative Ald1 protein was compared to database homologs (http://www.ncbi.nlm.nih.gov/blast), as a confirmation of the conceptually translated cDNA nucleotide sequence. The sequence analysis revealed that *ald1* encodes a putative polypeptide of 502 amino acid residues, which is interrupted by 16 introns (Fig. 8).

The comparison of Ald1 with other fungal ALDHs also shows that it clusters with specific fungal ALDHs with up to 74% amino acid identity, the closest ALDH being from *Laccaria bicolor* (Xp_001889968), which is also an ectomycorrhizal fungus (Fig. 9). Interestingly, Ald1 has an amino acid identity of 58% with *U. maydis* indole-3-acetaldehyde dehydrogenase, iad1, which is involved in production of IAA (Basse *et al*., 1996). The alignment of the fungal ALDHs revealed 10 highly conserved motifs, which were observed to cluster near the active site of the enzymes (Perozich *et al*., 1999). Also, 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 of ALDHs (Lie *et al*., 1997), and two sequences for the general glycine motif, GXGXXXG, for NAD(P) binding (Ferres *et al*., 1989; Jung and Lee, 2006), were identified (Fig. 9).

Most fungal ALDHs aligned with Ald1 show a relatively high similarity of the first 16 amino acids to Ald1 (Fig. 9). The ATG start codon is supported by the possible translational start site AAGCATG (Fig. 8), with the consensus sequence A/GNCATG for vertebrates, and strong bias to purines (As) at the -3 and -4 nucleotide position, typical of eukaryotic genes (Cavener and Ray, 1991).

Using the program ExPASy (http://www.expasy.org), the protein molecular weight was calculated to be 53.5 kDa, and the theoretical isoelectric point is 6.28. Protein localization in the cell was predicted, using the program WoLF PSORT (http://wolfpsort.org), to be cytosolic.

Fig. 8. Full length *ald1* gene. A. Diagramatic representation of *ald1* showing the ORF, interrupted by 16 introns, and conserved domains. B. Nucleotide and amino acid sequence of *ald1* and its flanking sequences. Five stress response elements (black boxes), two GC-box-like elements (blue boxes), two CAAT box-like elements (green boxes) and a TATA box-like element (red box) were putatively identified in the promoter region. The putative polyadenylation signal is shaded as a purple box in 3' non-coding sequence. Start and stop codons are underlined.

The analysis of the promoter region led to the prediction of five putative Stress Response Elements (STREs) with consensus sequence CCCCT or AGGGG (Fig. 8), which characterize stress-inducible genes (Martinez-Pastor *et al*., 1996; Görner *et al*., 1998; Moskvina *et al*., 1999; Zähringer *et al*., 2000). A putative TATA box, TATATATT, the possible core promoter sequence of *ald1*, which is consistent with TATA boxes of eukaryotes (Juo *et al*., 1996), was identified 179 bp upstream the transcription start codon. A search for a putative CAAT box, which binds the transcriptional factors CAAT transcriptional factor (CTF) and/or CAAT binding protein (CBP), yielded two sequences (TCAATGT and TCAATAT) adjacent to each other, upstream the TATA box (Fig. 8). Two putative GC boxes (consensus sequence GGGCGG), which bind the transcription factor Sp1 (reviewed in Dynan and Tjian, 1985), were identified upstream of TATA box and CAAT box. Thus, the promoter elements expected for a eukaryotic gene could be identified.

Downstream of the putative stop codon (TAA), the mRNA contains 525 bp of non-coding sequence as indicated by the cDNA sequence, i.e. 525 bp before the poly-A tail (Fig. 8). A putative polyadenylation signal (AATTAT), with a core consensus sequence AATAAA, is found 427 bp down stream from the stop codon. A search for nucleotide frequencies of the translation termination site common to eukaryotes revealed the presence of adenosine at position +1 (immediately downstream of the stop codon), which is the most preferred among eukaryotes (Cavener and Ray, 1991). Also, the downstream region of the putative polyadenylation signal is rich in T and G nucleotides, a feature shared by most eukaryotic mRNAs (Kim and Martinson, 2003).

Furthermore, adenosine is found in position +4, immediately after the stop codon, which conforms to the nucleotide frequency bias reported in Cavener and Ray (1991) that may be the result of selection for nucleotides that optimize termination of translation. The region +1 to +10 is AT rich (80%), which is the preferred condition for transcription/translation termination by mRNAs with TAA stop codons (Cavener and Ray, 1991).

3.1.3. Phylogenetic relationships between fungal aldehyde dehydrogenases

Using the full length translated amino acid sequence of Ald1, a phylogenetic relationship with other fungal ALDHs was reconstructed. The search for fungal ALDHs to be used in reconstructing phylogeny revealed ALDH sequences of fungal genomes that belong to the ALDH superfamily characterized by 10 conserved motifs indicated in Perozich *et al*. (1999). However, only a small part of the ALDH sequences clusters in the specific group of fungal ALDHs (Table 5). Sequences and GenBank accession numbers for *Phanerochaete*

chrysosporium, *Sporobolomyces roseus*, *Schizophyllum commune, Phycomyces blakesleeanus* and *Batrachochytrium dendrobatidis* can be found at http://genome.jgi-psf.org and those for *Cryptococcus neoformans* and *Puccinia graminis* at http://blast.jcvi.org and http://www.broadinstitute.org, respectively. The remaining sequences and their GenBank accession numbers can be found at http://www.ncbi.nlm.nih.gov. As the table indicates, all fungi, with the exception of the chytridiomycete *Batrachochytrium dendrobatidis*, are characterized by duplication of fungal ALDHs, with the basidiomycetes closely related to *T. vaccinum* possessing at least 3 paralogs.

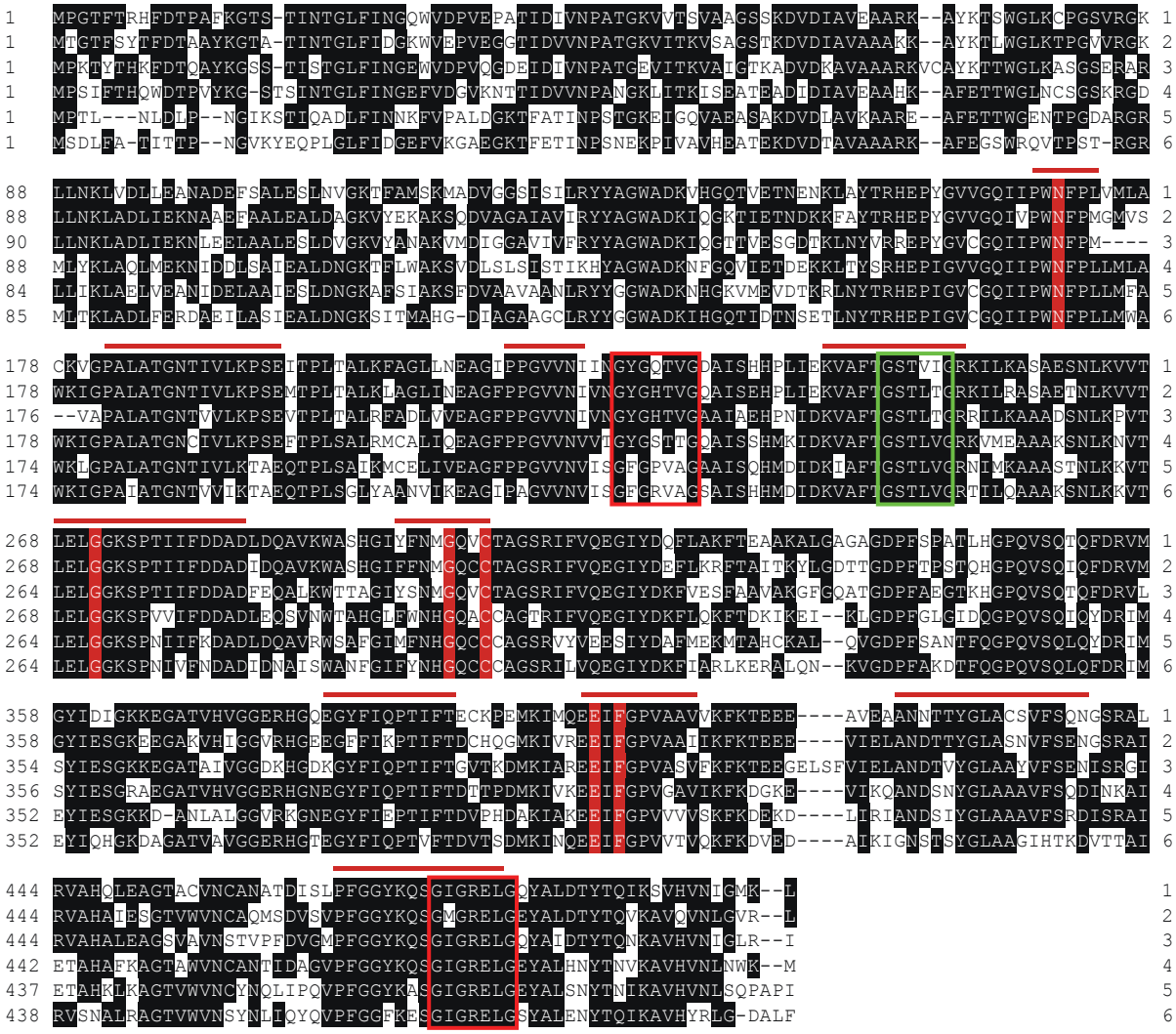


Fig. 9. Alignment of the deduced Ald1 amino acid sequence (1) with other fungal ALDH sequences: *Laccaria bicolor* (2: XP_001889968), *Coprinopsis cinerea* (3: XP_001834665), *Agaricus bisporus* (4: 074187), *Ustilago maydis* (5: XP_758655) and *Aspergillus niger* (6: P41751). The red horizontal lines represent conserved motifs while the conserved amino acid residues are shaded in red. One putative NAD-binding domain proposed for ALDHs (green box) and two general NAD(P)-binding domains (red boxes) were identified.

The widely observed duplication of fungal ALDHs prompted us to investigate the evolutionary origin of this enzyme family. Thus, fungal ALDH phylogeny was reconstructed under Bayesian inference using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) on MAFFT-aligned amino acid sequences. The resulting phylogram (Fig. 10) shows a well supported (high probability values) clustering of fungal ALDHs in two groups each of basidiomycota and ascomycota, with a chytridiomycota ALDH being basal to all other fungal ALDHs, agreeing with the current tree of life (Blackwell *et al*., 2009). The 3 ALDH sequences from a zygomycete *Phycomyces blakesleeanus* clustered in two different clades, with ascomycetes, surprisingly, being basal to them.

Table 5. Aldehyde dehydrogenase enzymes retrieved from fungal genomes of basidiomycota, ascomycota, zygomycota and chytridiomycota

Fungal genome	ALDH superfamily homologs	Fungal ALDH homologs
Laccaria bicolor	15	3
Coprinopsis cinerea okayama	14	4
Phanerochaete chrysosporium	20	7
Schizophyllum commune	16	3
Ustilago maydis	18	3
Cryptococcus neoformans	12	2
Puccinia graminis	10	2
Sporobolomyces roseus	10	2
Aspergillus nidulans	26	3
Neurospora crassa	13	2
Candida albicans	12	2
Saccharomyces cerevisiae	5	3
Schizosaccharomyces pombe	5	2
Phycomyces blakesleeanus	9	3
Batrachochytrium dendrobatidis	3	1

As shown in the phylogram, 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 *Batrachochytrium dendrobatidis*. The second major duplication event happened within the basidiomycota 1 group (Fig. 10), primarily in the agaricomycetes clade. Interestingly, Ald1 clustered together with the other two *T. vaccinum* ALDH partial sequences, Ald2 and Ald3, earlier identified by Krause (2005). The three ALDHs from *T. vaccinum* clustered with other agaricomycetes of the basidiomycota 1 group, but formed a distinct, fully supported branch typical for a duplication after the separation of this species from others. Multiple fungal ALDH sequence alignments had indicated that *Coprinopsis cinerea* Ald4, which clusters in

the basidiomycota 1 group (Fig. 10), had lost many amino acids, especially from the essential conserved motifs (Perozich *et al*., 1999), probably explaining why it has a long branch. Further phylogenetic analyses using Treefinder, RAxML and PAUP confirmed these results since similar topologies were generated (data not shown).

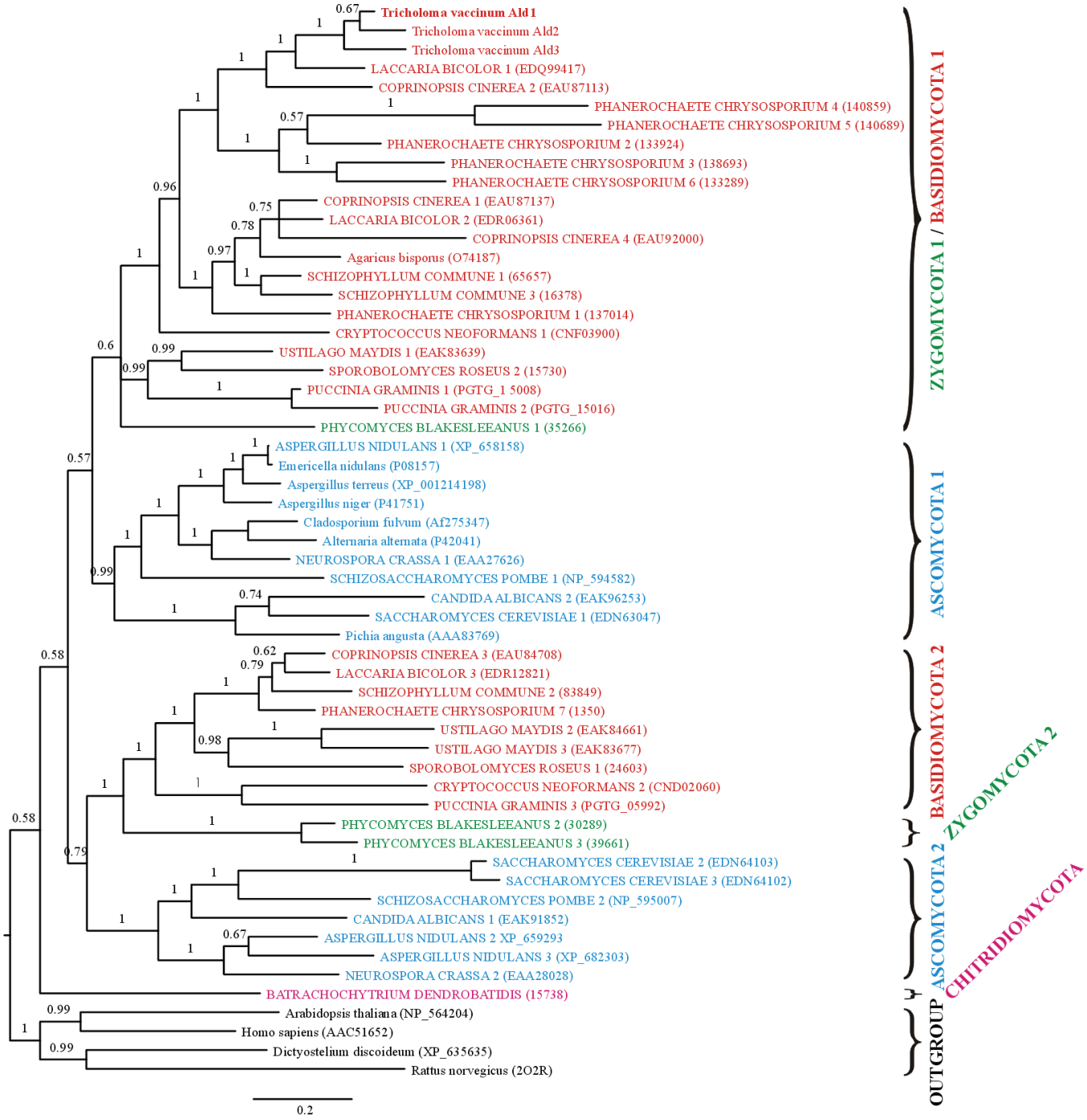


Fig. 10. A semistrict consensus phylogram of fungal aldehyde dehydrogenase enzymes (ALDHs), created with MrBayes 3.1.2. Bayesian posterior probability values are shown above corresponding branches. Branch lengths are proportional to evolutionary distances. Mammalian, plant and mycetozoa ALDHs were included as an outgroup. The first single digit number immediately in front of a binomial represents the i^{th} ALDH sequence of a given species, in a descending order of similarity to Ald1. After the binomials, GeneBank accession numbers (in brackets) are given. Binomials in capitals indicate availability of their genome.

3.1.4. Verification of *ald1* cDNA by heterologous expression in *E. coli*

To confirm the integrity of the cloned *ald1* gene, its cDNA was heterologously expressed in *E. coli* using the pET-expression system (Invitrogen). The *ald1* expression was then verified by electrophoresis and enzyme activity assays using crude soluble protein extracts.

SDS-PAGE analysis of crude soluble protein extracts, stained with Coomassie Brilliant Blue R250 solution, did not reveal significant gene expression differences between the 0.9 mM IPTG-induced and uninduced *E. coli* cells (Fig. 11A). Therefore, to specifically detect only Ald1 protein and reduce the background, SDS-PAGE gels were subjected to Western blot analysis targeting the C-terminal His-tag fused to Ald1 in the pET-expression system. The results show that Ald1, with an apparent molecular mass of approximately 53 kDa, was expressed (Fig. 11B). This protein size correctly corresponds to the calculated (http://www.expasy.org/tools) size of 53.5 kDa. However, as observed in Coomassie-stained SDS-PAGE gels, there was no significant induction by the used concentration of IPTG since there was no enrichment of the protein band corresponding to Ald1, with crude proteins extracted from induced *E.coli* cells compared to uninduced cells (Fig. 11B). In addition, a second band of a smaller size (approximately 45 kDa) was observed, which might indicate proteolysis.

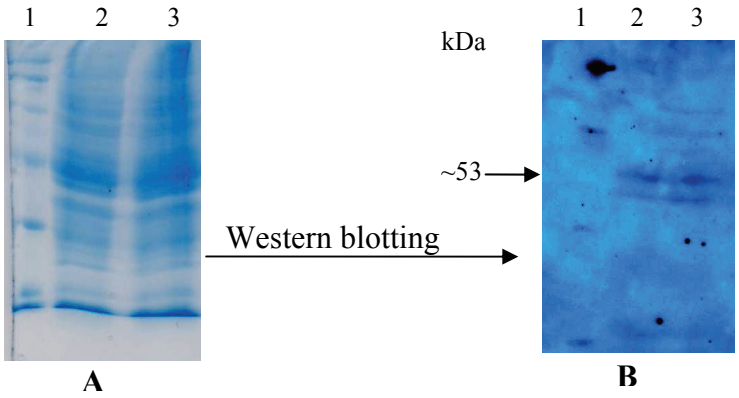


Fig. 11. SDS-PAGE (A) and Western blot (B) analysis of Ald1 protein. Crude soluble protein extracts from *tvaldh1*-expressing *E. coli* cells were analyzed. Lane 1 His-tagged molecular mass standard, Rotimark standard (Roth), lane 2 protein extracts from IPTG-induced *E.coli*, lane 3 protein extracts from uninduced *E.coli*.

The enzymatic activity, substrate specificity and cofactors were tested using crude soluble protein extracts of the *E. coli* cells expressing *ald1*. The activity was assayed spectrophotometrically by monitoring the formation of NAD(P)H. Ald1 oxidized short chain aliphatic aldehydes notably propionaldehyde and, to some extent, butyraldehyde (Tables 6

and 7). Although there was no enzyme activity detected with the tested aromatic aldehydes, the data with indole-3-acetaldehyde as a substrate seem to suggest that Ald1 may also, at least to some extent, oxidize it because the reaction showed consistently higher absorbance than the control, albeit loss of activity with time (data not shown). This made it impossible to calculate change in relative absorbance per unit time, which was used as the measure for enzyme activity (Tables 6 and 7). Furthermore, despite the fact that the results indicate that acetaldehyde is not a substrate for Ald1, this cannot be confirmed since the extremely volatile aldehyde made it impossible to conclude the enzyme activity assay for it.

Since both NAD and NADP 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^+. As expected, Ald1 exhibited no enzyme activity on ethanol.

Table 6. Enzyme activity and substrate specificity of Ald1: β-NAD^+ as a cofactor

Substrate	**Enzyme activity (**change in relative absorbance/min x10^{-4})
Propionaldehyde	1.1
Butyraldehyde	2.7
Acetaldehyde	n.d**
Benzaldehyde	0.0
Indole-3-acetaldehyde	n.d*
Ethanol	0.0

n.d: not detected
* Absorbance higher than in controls, but declined over time
** Enzyme activity, using acetaldehyde as a substrate, was not conclusive due to its high volatility

Table 7. Enzyme activity and substrate specificity of Ald1: β-$NADP^+$ as a cofactor

Substrate	**Enzyme activity (**change in relative absorbance/min x10^{-4})
Propionaldehyde	13.1
Butyraldehyde	0.0
Acetaldehyde	n.d**
Benzaldehyde	0.0
Indole-3-acetaldehyde	n.d*
Ethanol	0.0

n.d: not detected
* Absorbance higher than in controls, but declined over time
** Enzyme activity, using acetaldehyde as a substrate, was not conclusive due to its high volatility

3.1.5. Induction of *ald1* expression by alcohol and aldehyde-related stress

The regulation of *ald1* expression, as mediated by various aldehydes and ethanol, was investigated using real time RT-PCR. First, aldRTPCRF and aldRTPCRR primers were used

to generate a standard curve using different concentrations of *ald1* cDNA cloned in pDrive (Fig. 12B). The expected 132 bp real time RT-PCR amplicon (Fig. 12A) was monitored in amplification plots (Fig.13) for amplification artifacts. The standard curve was used to calculate the *ald1* transcript levels in aldehyde- and ethanol-treated fungal cultures.

The results show that 0.1 mM Indole-3-acetaldehyde and benzaldehyde significantly induced *ald1* transcription (Fig. 14), with about 3 fold and 4 fold transcript accumulation, respectively (Table 8). However, a concentration of 0.01 mM Indole-3-acetaldehyde was probably too low to significantly increase the *ald1* mRNA amount. The observation of treatments with ethanol revealed a concentration-dependent *ald1* transcriptional inhibition, with 0.01% ethanol showing about 4 fold relative transcript accumulation compared to 1 fold of 0.1% ethanol (Table 8), probably suggesting ethanol toxicity as, in fact, reflected in the reduced biomass with 0.1% ethanol concentration. The results, nonetheless, indicate that low, most likely physiologically relevant ethanol concentrations strongly induce *ald1* transcription (Fig. 14).

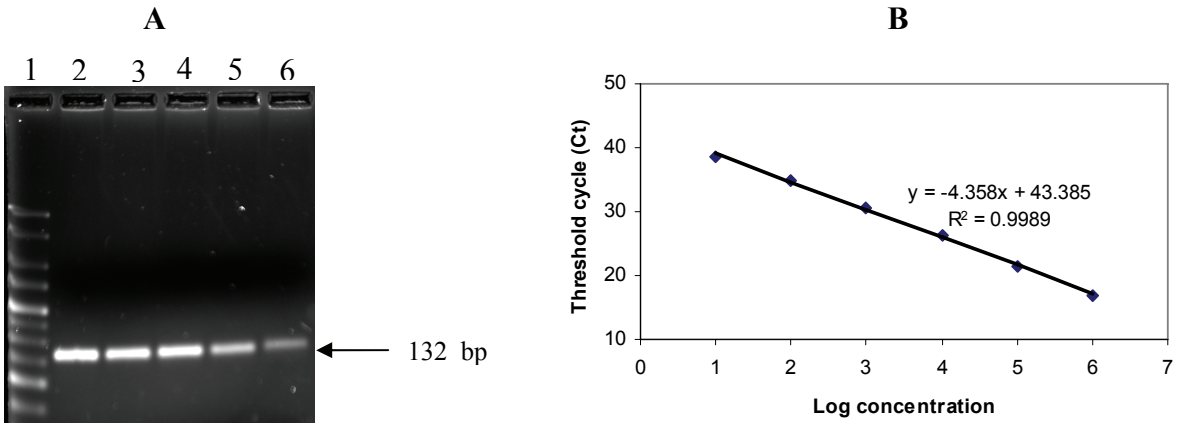


Fig. 12. Real time RT-PCR standard curve generation for quantification of *ald1* mRNA levels. A shows PCR products, which were run on 3.5% agarose gel to visualize the expected 132 bp *ald1* cDNA fragment. A molecular ruler Hyperladder V (Bioline) (Lane 1) was used to estimate the fragment size. Lanes 2-6 represent 1000 pg, 100 pg, 10 pg, 1 pg and 0.1 pg plasmid DNA of *ald1* cDNA clone respectively. B shows the standard curve for quantifying *ald1* mRNA levels in samples.

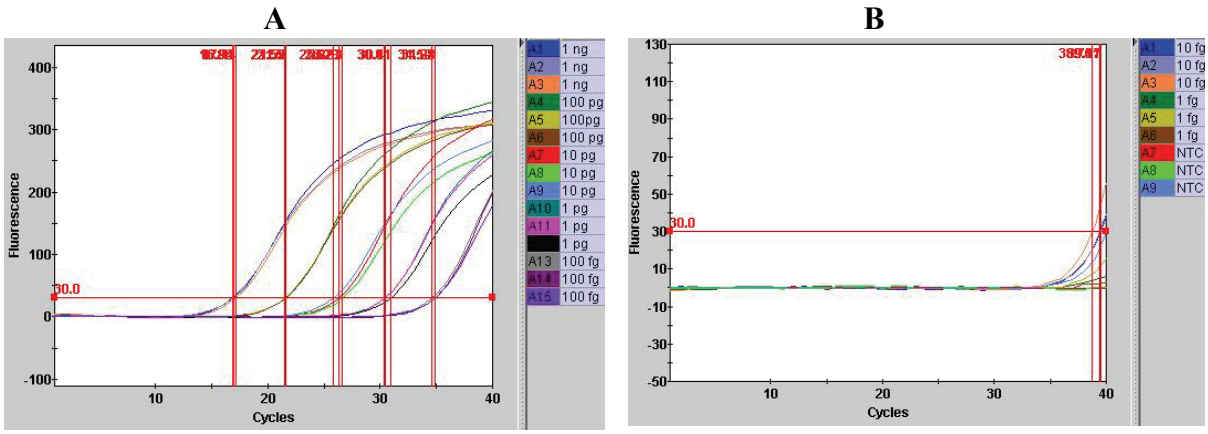


Fig. 13. Amplification plots of *ald1* cDNA Real time RT-PCR. Different concentrations of plasmid DNA with *ald1* cDNA fragment were used as template: 1 ng – 100 fg (A) and 10 fg-1 fg (B). Non-template control (NTC), which was nucleic acid free water (Fermentas, Germany), was used. Threshold fluorescence was set at 30. Three PCR reactions per concentration were used.

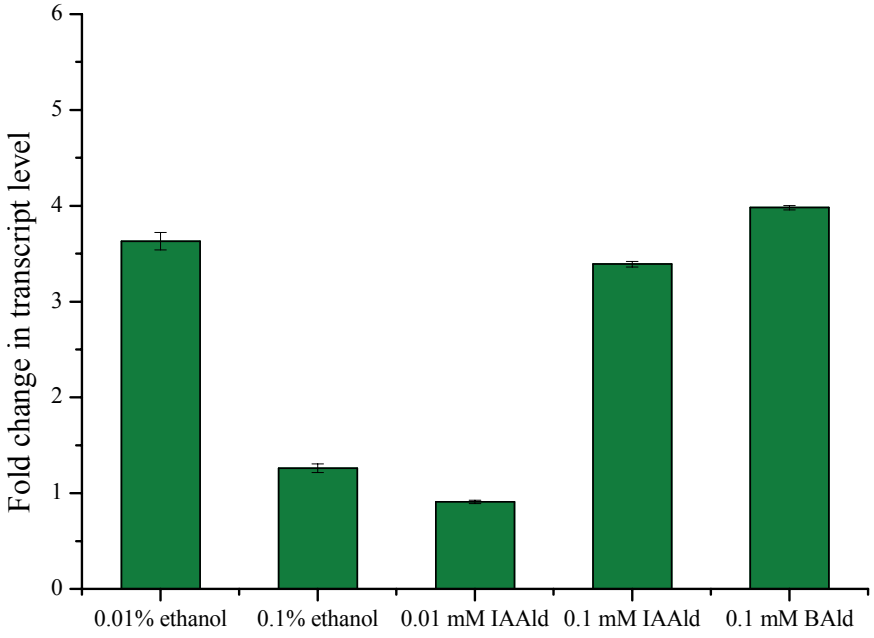


Fig. 14. Ethanol and aldehyde-mediated *ald1* transcription induction. RNA for Real time RT-PCR was extracted from fungal cultures incubated with 0.01% and 0.1% ethanol, 0.01 mM and 0.1 mM IAAld, and 0.1 mM benzaldehyde (BAld). Each treatment had 2 replicates. The PCR was carried out on 2 cDNA replicates in 3 reactions per cDNA replicate. Bars denote standard error.

Table 8. Effect of ethanol and aldehyde stress on *ald1* transcript accumulation

Treatment	Relative transcript level
0.01% ethanol	4
0.1% ethanol	1
0.01 mM indole-3-acetaldehyde	1
0.1 mM indole-3-acetaldehyde	3
0.1 mM benzaldehyde	4

3.2. Analysis of *ald1* gene function by overexpression in *T. vaccinum*

3.2.1. Construction of *ald1* gene overexpression vectors

In order to analyze the possible function of *ald1* gene, two plasmids carrying *ald1*-overexpressing cassettes, one with *gpd* promoter and 35S terminator, designated pBGaldh1 (Fig. 15A), and the other with the same features but with an additional *egfp* fusion, designated pBGaldh1SeGFP (Fig.16A), were constructed. Both plasmids consist of a pCAMBIA 1300 backbone containing the kanamycin and hygromycin resistance genes. First, different PCRs were carried out to generate fragments for the constructs. Using the forward primer gpdF, with *SacI* restriction site at the 5' end, and the reverse primer gpdR on pBGgHg plasmid DNA as a template, an approximately 280 bp fragment of the constitutive strong glyceraldehyde-3-phosphate dehydrogenase promoter (P*gpd*) from *A. bisporus* was obtained. Next, *ald1* was amplified with the ORFF forward and ORFR reverse (*AscI* restriction site at the 5' end) primer pair on genomic *T. vaccinum* DNA. A fusion PCR was subsequently 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 by using pBGgHg plasmid DNA as a template with EGFPFII forward (*Asc*I restriction site at 5' end) and EGFPRII reverse (*Pme*I restriction site at 5' end) primer pair. A 35S fragment of 340 bp was generated by a PCR on the same plasmid DNA template using 35SF forward (*Asc*I restriction site at 5' end) and EGFPRII reverse primers.

Then, series of ligations of the PCR fragments generated in different plasmids followed by restrictions and re-ligations generated the final binary vectors for *ald1* overexpression. To construct plasmid pBGaldh1 (Fig. 15A), intermediate plasmid pNGaldh1 was generated by excising the *Sac*I-*Asc*I fragment from a commercial vector pNEB193 and inserting the P*gpd-ald1* PCR product *via Sac*I-*Asc*I ligation. Plasmid pNGaldh1 was then restricted with *Asc*I and *Pme*I, followed by insertion of the 35S terminator PCR fragment to generate plasmid pNGaldh1S. The correct P*gpd-ald1*-35S construct (Fig. 15B) was confirmed both by PCR and

restriction analyses. The entire P*gpd-egfp*-35S fragment was excised from plasmid pBGgHg by *Sac*I and *Pme*I and replaced by the P*gpd-ald1*-35S excised from pNGaldh1S by the same restriction enzyme combination to obtain plasmid pBGaldh1. Again, P*gpd-ald1*-35S construct integration into this plasmid was confirmed by PCR and restriction analyses (Fig. 15C). Since this was the destination vector for overexpression, the correct nucleotide sequence and orientation of the construct was confirmed by sequencing.

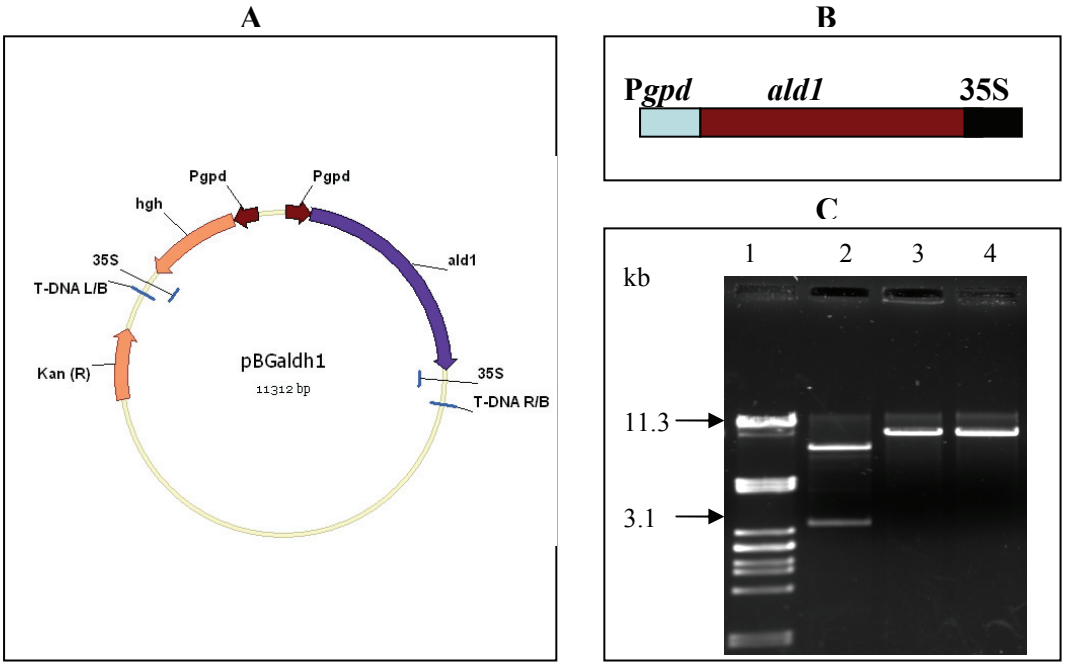


Fig. 15. Construction of *ald1* overexpression plasmid pBGaldh1. pBGaldh1 (A) is 11.312 kb in size and consists of a pCAMBIA 1300 backbone containing the kanamycin resistance gene and the right border (R/B) and left border (L/B) sequences of *A. tumefaciens* T-DNA. The hygromycin resistance gene and *ald1* gene, introduced in the plasmid by insertion of a 3.1 kb Pgpd-*ald1*-35S (T) cassette (B), are located between the border sequences, and each is joined to the constitutive strong *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase promoter (Pgpd) and cauliflower mosaic virus terminator (35S). Correct cloning of Pgpd-*ald1*-35S construct into the vector was confirmed by, among others, restriction analysis (C). Lane 1 shows DNA size marker *λPst*I. Plasmid pBGaldh1 restriction by *Sac*I and *Pme*I releases the 3.1 kb construct from the plasmid (lane 2) while *Sac*I (lane 3) and *Pme*I (lane 4) linearize the plasmid.

Plasmid pBGaldh1SeGFP (Fig. 16A) was constructed by first restricting plasmid pNGaldh1 with *Asc*I and *Pme*I followed by insertion of the *egfp*-35S PCR fragment to generate the plasmid pNGaldh1gfp. Both PCR and restriction analysis confirmed the correct cloning of P*gpd-ald1-egfp*-35S (Fig. 16B). The entire P*gpd-egfp*-35S fragment was excised from plasmid pBGgHg by *Sac*I and *Pme*I and replaced by the P*gpd-ald1-egfp*-35S fragment excised from pNGaldh1gfp by the same restriction enzyme combination to obtain plasmid pBGaldh1SeGFP. Again, P*gpd-ald1-egfp*-35S integration was confirmed by PCR, restriction analysis (Fig. 16C) and sequencing.

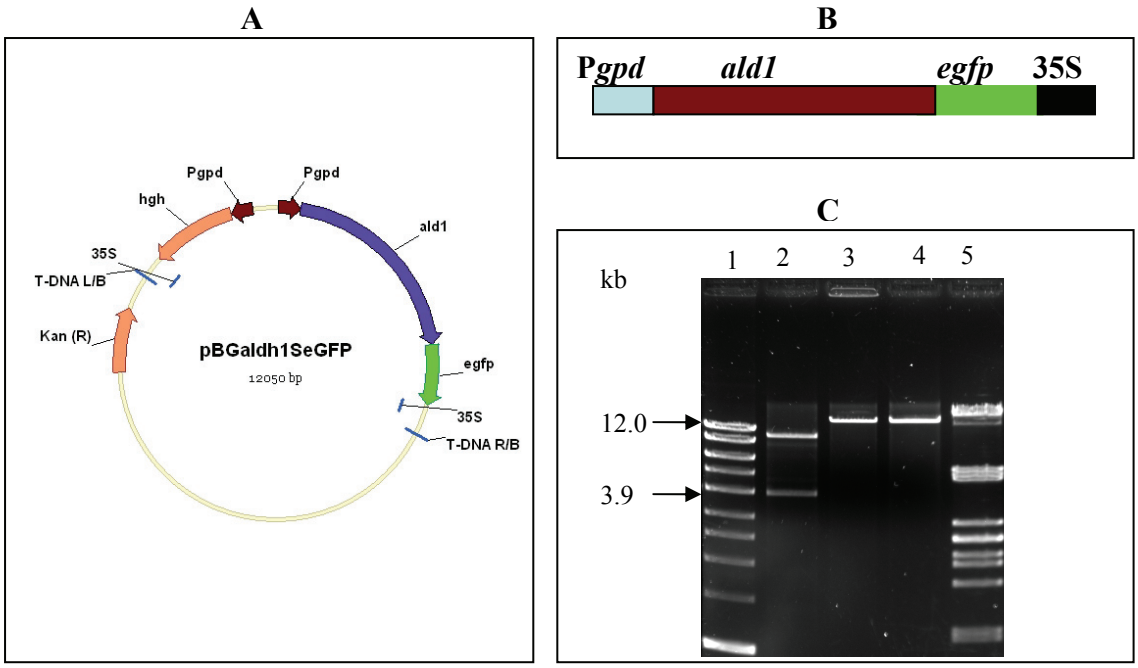


Fig. 16. Generation of *ald1* overexpression plasmid pBGaldh1SeGFP, with *egfp* fusion. pBGaldh1SeGFP (A) is 12. 050 kb in size and consists of a pCAMBIA 1300 backbone containing the kanamycin resistance gene and the right border (R/B) and left border (L/B) sequences of *A. tumefaciens* T-DNA. The hygromycin resistance gene and *ald1* gene, introduced in the plasmid by insertion of a 3.9 kb P*gpd-ald1-egfp*-35S (T) cassette (B), are located between the border sequences, and each is joined to the constitutive strong *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase promoter (P*gpd*) and cauliflower mosaic virus terminator (35S (T)). Correct cloning of Pgpd-*ald1-egfp*-35S (T) construct into the vector was confirmed by, among others, restriction analysis (C). Lane 1 shows "high range DNA ladder" (Bioline) and lane 5 the marker *λPst*I. Plasmid pBGaldh1SeGFP restriction by *Sac*I and *Pme*I releases the 3.9 kb construct from the plasmid (lane 2) while *Sac*I (lane 3) and *Pme*I (lane 4) linearize the plasmid.

3.2.2. *Agrobacterium tumefaciens*-mediated transformation of *T. vaccinum*

T. vaccinum was transformed with *A. tumefaciens* strain AGL-1 carrying plasmids pBGaldh1 (Fig. 15A) or pBGaldh1SeGFP (Fig. 16A), which were constructed to overexpress *ald1*. After co-cultivation for about 2 weeks on selection plates, putative hygromycin resistant fungal transformants were observed. PCR analysis, using primers Hph-F and Hph-R spanning the hygromycin resistance gene (*hgh*), on DNA isolated from the putative transformants confirmed the presence of *hgh* both in the $Hyg^r/eGFP^+$ and $Hyg^r/eGFP^-$ transformants, while this DNA segment was not detected in wildtype (Fig. 17A). PCR analysis with Egfp-1 and Egfp2 spanning the *egfp* gene confirmed the presence of *egfp* in $Hyg^r/eGFP^+$ transformants, which was absent in wildtype (Fig. 17 B). These results indicated that the T-DNA regions of the transforming plasmids, which included *hgh* and *egfp* genes as a selection marker and a reporter gene, respectively, were integrated into *T. vaccinum* genome.

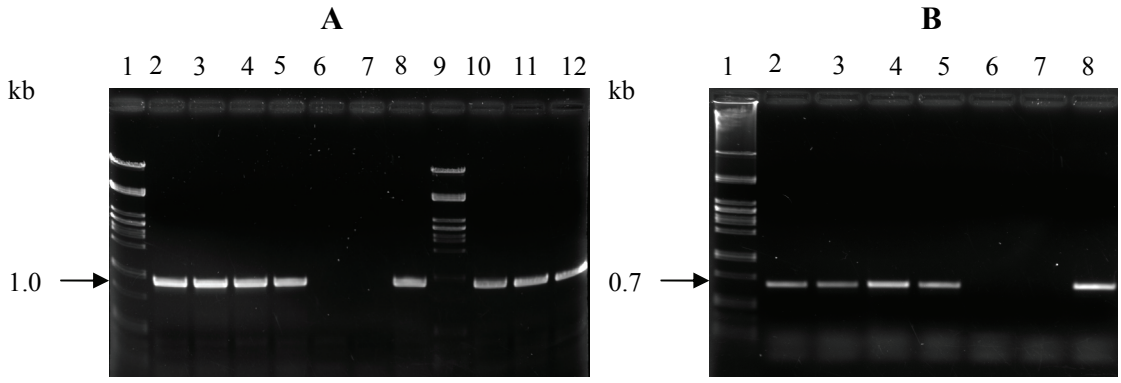


Fig. 17. PCR analysis of *T. vaccinum* transformants. PCR targeting the 1.0 kb *hgh* gene (A) was carried out both on transformants with or without *egfp* gene fusion: Lanes 1 and 9 DNA size maker *λPst*1, lanes 2-5 $Hyg^r/eGFP^+$ transformants, lane 6 wild type, lane 7 negative control without DNA, lane 8 positive control with vector pBGgHg, lanes 10-12 $Hyg^r/eGFP^-$ transformants. PCR targeting the 0.7 kb *egfp* gene (B) was carried out only on $Hyg^r/eGFP^+$ transformants: Lane 1 DNA size maker *λPst*1, lanes 2-5 $Hyg^r/eGFP^+$ transformants, lane 6 wild type, lane 7 negative control without DNA, lane 8 positive control with vector pBGgHg. PCR was carried out on gDNA of the transformants.

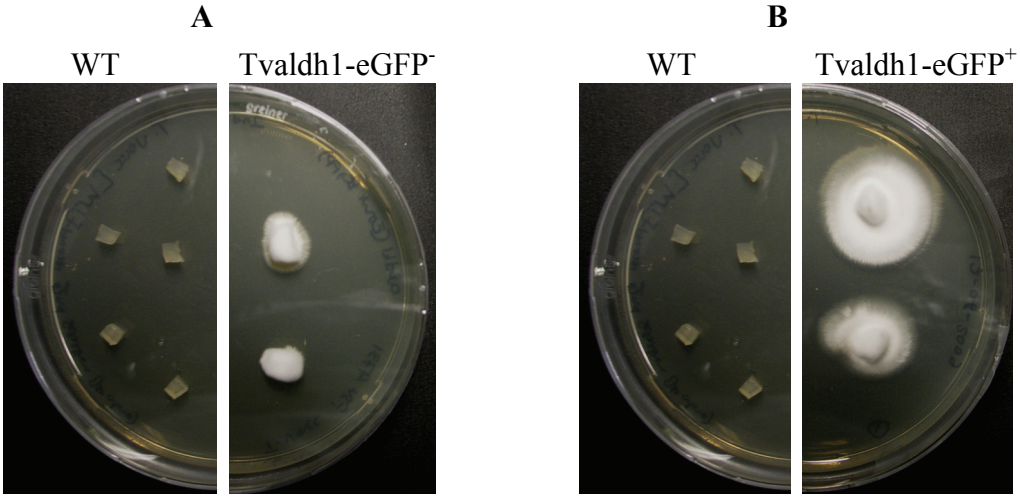


Fig. 18. Hygromycin resistant *T. vaccinum* transformants observed on plates after the second round of antibiotic selection. *T. vaccinum* was transformed with *A. tumefaciens* AGL-1 carrying the transforming plasmids pBGaldh1 without *egfp* gene fusion (panel A) and pBGaldh1SeGFP with *egfp* gene fusion (panel B). A: Non-transformed *T. vaccinum* wild type (WT) failed to grow on selection plates while Tvaldh1-$eGFP^-$ transformants grew, albeit slowly. B: Tvaldh1-$eGFP^+$ transformants established good growth on selective plates as compared to the wild type *T. vaccinum* that failed to grow. Cultures were grown on MMNb-medium supplemented with 25 µg/ml hygromycin B.

The positive transformants were transferred to fresh selection agar plates of MMNb-medium containing 25 µg/ml hygromycin B for the second round of antibiotic selection. Hygromycin resistant transformants, observed on selection plates after the second round of antibiotic selection (Fig. 18), were transferred to non-selective MMNb-medium for mitotic stability tests.

For mitotic stability tests, transformants were grown on non-selective MMNb-plates for about 2 months before transferring them back to selective plates containing 25 µg/ml Hygromycin B, and evaluating their growth. All transformants were still able to grow well on hygromycin plates (Fig. 19), indicating that the introduced *hgh* gene is mitotically stable.

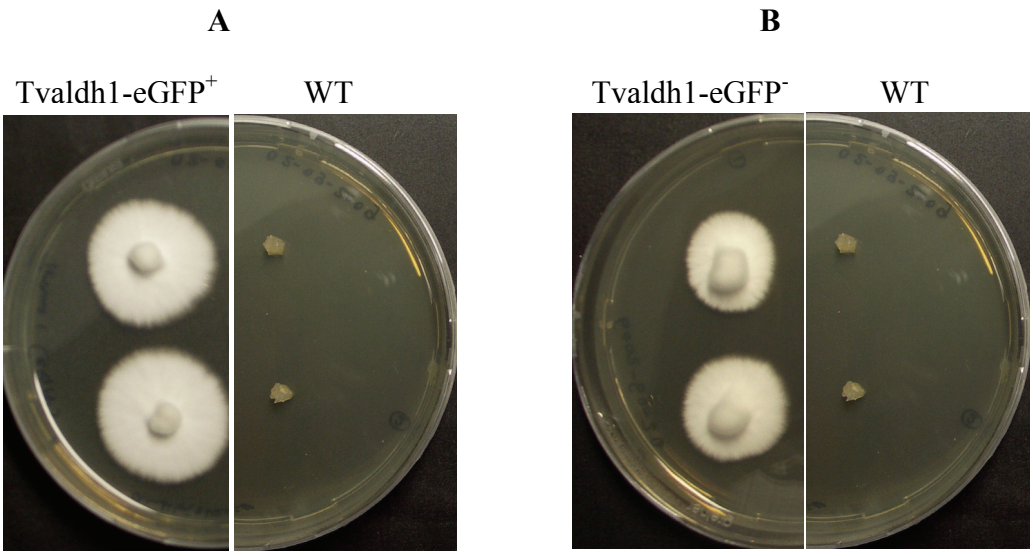


Fig. 19. Mitotic stability test for *T. vaccinum* transformants. After approximately 2 months growth on non-selective plates, the growth of transformants on selective plates containing hygromycin B was monitored. Both the transformants with (A) and without (B) *egfp* gene fusion maintained their growth on the selective plates while no wild type fungal growth was observed. The transformants were grown on MMNb-medium supplemented with 25 µg/ml hygromycin B.

In order to investigate the correct functional expression of the *egfp*, mycelium was detached from colonies of transformants and analyzed for eGFP-associated green fluorescence by confocal laser-scanning microscopy. Preliminary confocal microscopy indicated that green fluorescence, associated with eGFP, was well distributed in the fungal hyhae of transformants, with significantly higher accumulation in specific regions (Fig. 20). A closer observation of different specimens indicated that the eGFP-associated green fluorescence was mostly confined to cytoplasm. Unfortunately, efforts to quantify eGFP-associated fluorescence of the transformants relative to wild type strain later on in sub-cultured transformants yielded no significant difference in green fluorescence to the wildtype (data not shown). These data showed that the green fluorescence in mycelium of transformants was drastically reduced, as compared to the first confocal images taken.

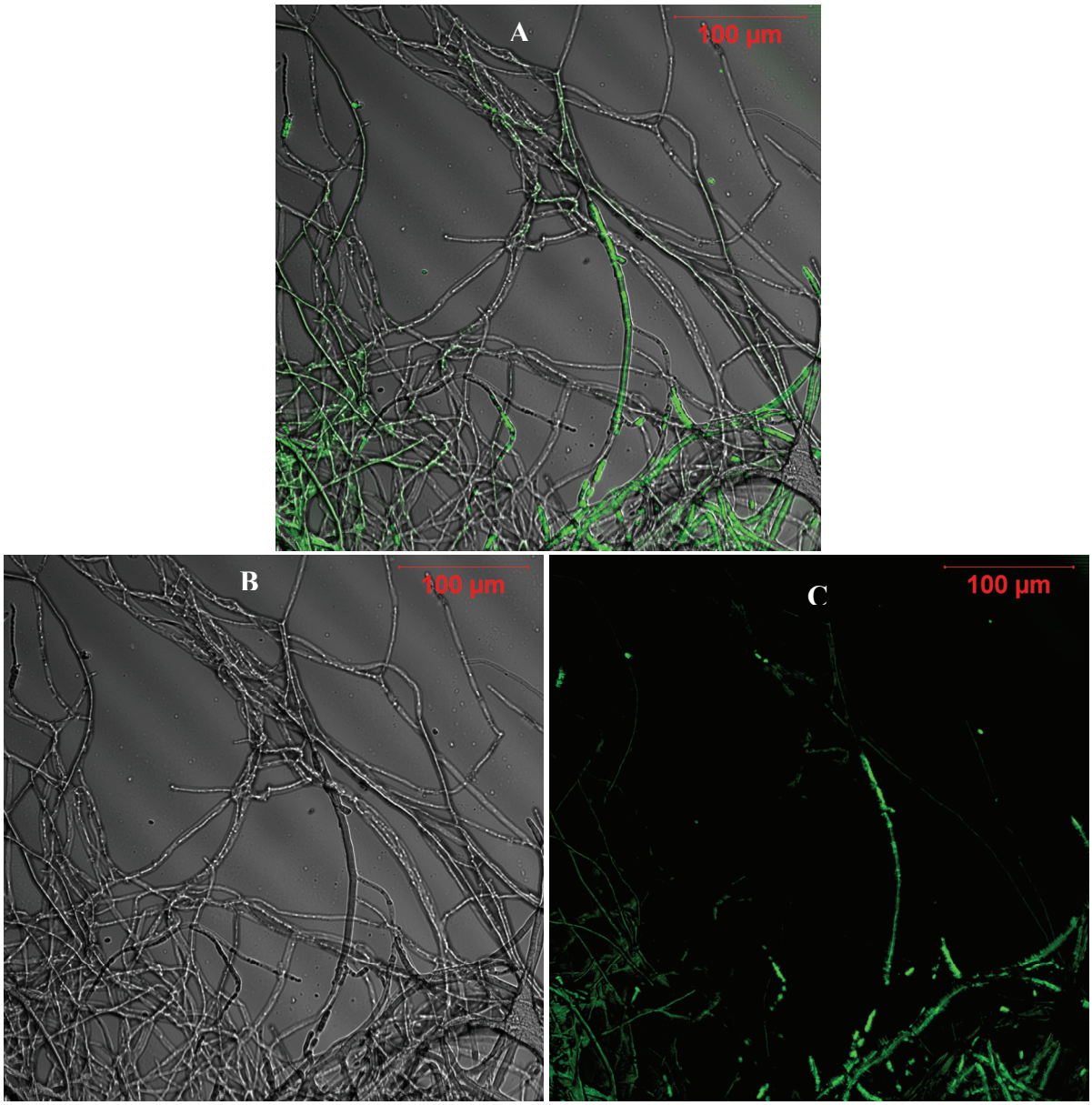


Fig. 20. Analysis of eGFP-associated green fluorescence in transgenic *T. vaccinum*, transformed with an *egfp* fusion plasmid pBGaldh1gfp, by laser-scanning confocal microscopy. (B) Detached mycelium visualized using transmitted light. (C) The same field showing green fluorescence due to eGFP accumulation in mycelium. (A) The two images were superimposed. The green fluorescence associated with eGFP was detected using 488 nm excitation and 505 nm long pass (LP 505) emission. Images were obtained with a 40x water objective.

3.2.3. *In vitro* mitigation of stress by *ald1*

Aldehyde dehydrogenase is widely known for its role in maintaining low levels of toxic aldehydes in organisms by oxidizing them to non-toxic, usable carbohydrates. To test detoxification ability of ALDH in ectomycorrhiza, *T. vaccinum* transformant (Tvaldh1-eGFP^{+}1) overexpressing *ald1* was investigated for ethanol and aldehyde stress torelance. First, toxicity tests were carried out using different concentrations of ethanol and aldehydes to determine the optimal concentrations for investigating the function of the fungal ALDH in

stress mitigation (data not shown). Concentrations of ethanol below 2% did not significantly slow down fungal growth; in fact, at lower concentrations, fungal strains showed increased growth by producing a dense network of aerial mycelium, probably as a result of using the non-inhibitory concentrations as alternative energy source. 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 wildtype (Fig. 21). Whereas the ethanol stress reduced growth of the wildtype by almost 50%, the growth of the transformant was only reduced by about 26-33% throughout the growth period of about 4 weeks (Fig. 21).

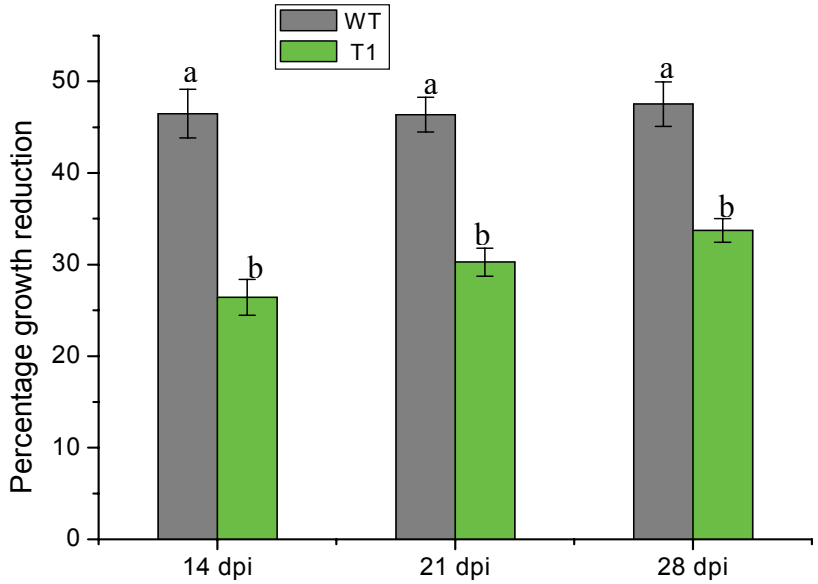


Fig. 21. Ethanol stress mitigation by *ald1*. The reduction in fungal growth, quantified using colony diameter measurements, as a result of exogenous application of 2% ethanol to fungal agar cultures was compared between the wild type *T. vaccinum* (WT) and a transformant overexpressing *ald1* gene with *egfp* fusion T1 (Tvaldh1-eGFP^{+}1). The fungal cultures were replicated at least 3 times. Bars denote standard error, and letters show significantly different fungal growths.

3.3. Role of indole-3-acetic acid in biology of *T. vaccinum*

A fundamental question about the involvement of IAA in ectomycorrhiza, which has not been investigated so far, is the effect of the phytohormone on the biology of ectomycorrhizal fungi.

This question was answered in this study. Furthermore, the capacity of *T. vaccinum* to produce IAA, a beneficial trait in ectomycorrhiza, was investigated.

3.3.1. Effect of IAA on *T. vaccinum* hyphal morphology

The effect of exogenously applied IAA or its precursors on fungal hyphal ramification was investigated *in vitro* by supplementing the liquid medium with varying concentrations of synthetic IAA or its precursors, and microscopically monitoring fungal morphology. Inoculation with the immediate IAA precursor, indole-3-acetaldehyde (IAAld), resulted in significant increase in fungal hyphal branching at precursor concentrations above 0.05 mM (Fig. 22). This effect was more pronounced at 0.2 mM IAAld, which was the highest concentration tested. The effect of another precursor, tryptophan, on branching was also tested. Tryptophan concentrations above 0.5 mM significantly increased hyphal branching, with highest branch formation becoming visible at 2.5 mM (Fig. 22).

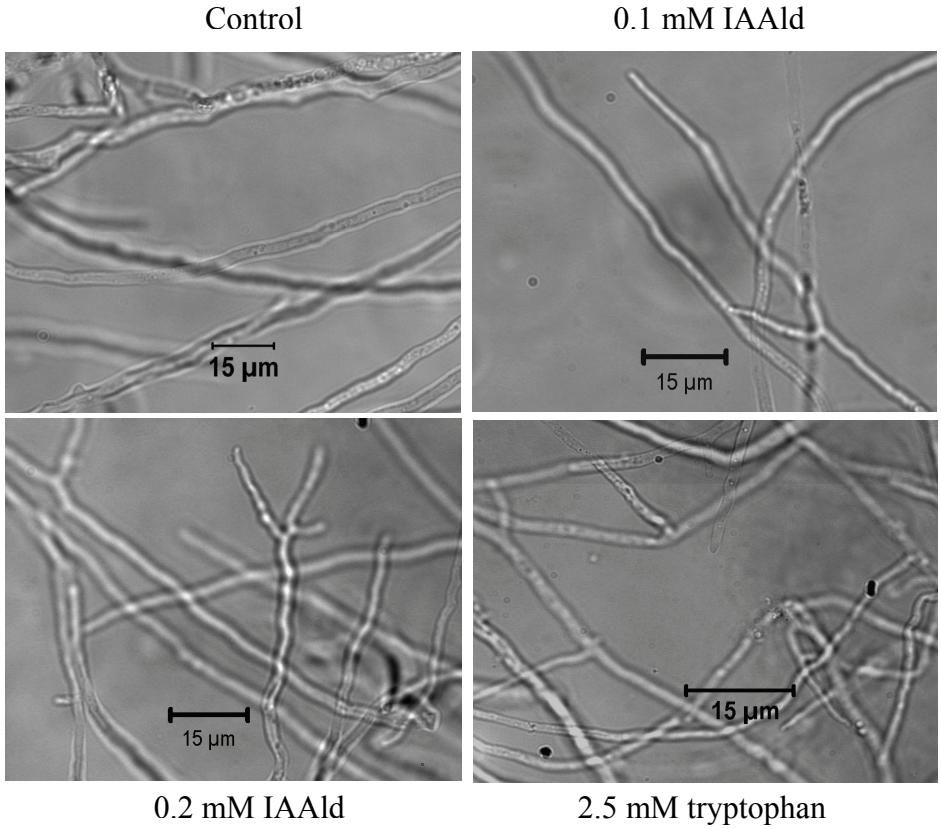


Fig. 22. *T. vaccinum* hyphal branching, as influenced by IAA precursors. Exogenous application of the IAA precursors Indole-3-acetaldehyde (IAAld) and tryptophan caused extensive fungal hyphal branching compared to the control where the fungal growth medium was not supplemented with the precursors, which showed almost no branching.

To find out if the hyphal phenotype observed was a result of IAA produced from the precursors, IAA concentrations in fungal culture filtrates were determined by the calorimetric Salkowski assay. Indeed, the assay showed that the fungus produced IAA from the precursors in a concentration-dependent manner (Fig. 23 and 24), which in turn correlated with the observed increased fungal hyphal branching. Although the results with IAAld as the precursor indicate that IAA concentration is reduced over time, an unsurprising observation since the phytohormone is unstable, the differences in phytohormone concentrations for different precursor concentrations are still discernable, even after 4 weeks of fungal culture incubations (Fig. 23). Importantly, the calorimetric IAA measurements suggest that *T. vaccinum* is able to produce IAA both in the presence and absence of exogenously supplied precursors, albeit with reduced concentrations in the latter case.

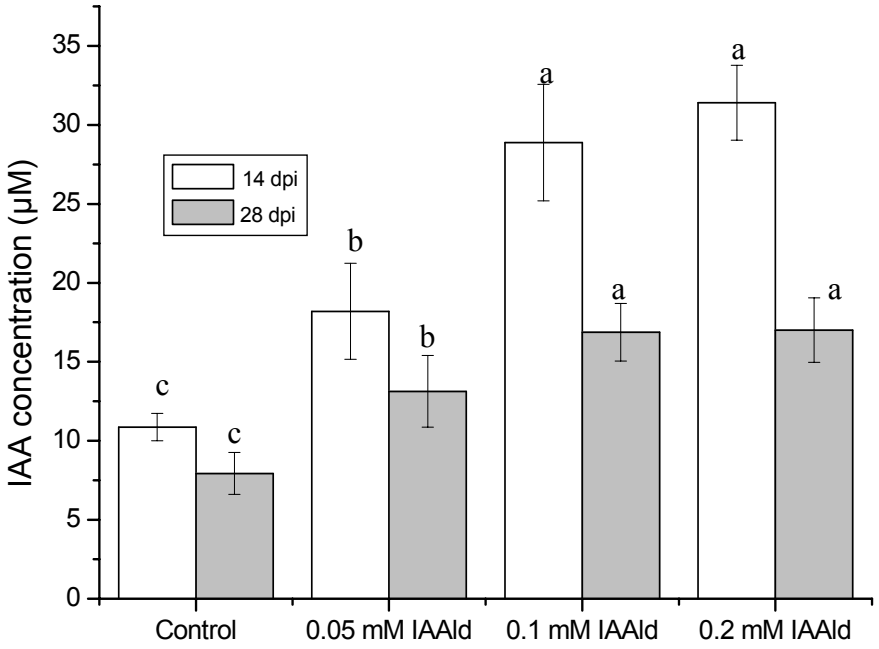


Fig. 23. IAA concentrations in fungal culture filtrates containing indole-3-acetaldehyde (IAAld). Concentrations were measured, in 4 week-old cultures, using Salkowski assay. The fungal cultures were replicated at least 4 times. Bars denote standard error, and letters show significantly different treatments.

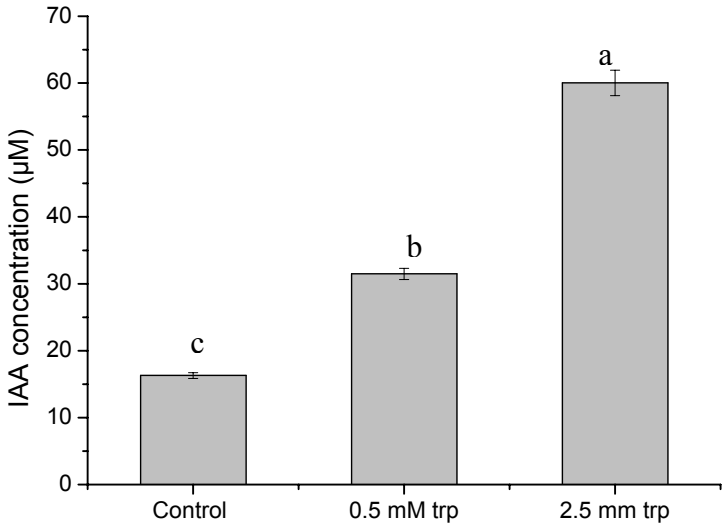


Fig. 24. IAA concentrations in fungal culture filtrates containing tryptophan (trp). Concentrations were measured using Salkowski assay in the 4 week-old cultures. The fungal cultures were replicated at least 4 times. Bars denote standard error, and letters show significantly different treatments.

To confirm the observation that IAA may be responsible for the increased hyphal branching with precursor treatments, pure synthetic IAA was exogenously applied to fungal cultures, and hyphal branching was monitored. Supplementation with 0.5 mM IAA provoked extensive hyphal branching (Fig. 25 B), although 0.1 mM of the phytohormone was enough to already significantly increase branching compared to cultures not supplemented (Fig. 25 A). IAA polar transport is known to be inhibited by different compounds including 2,3,5-triiodobenzoic acid (TIBA). To test if the exogenously supplied IAA was transported into the fungal cells, cultures were supplemented with a mixture of 0.5 mM IAA and 10 µM TIBA. Microscopic observations indicated that addition of TIBA reversed the phenotype observed with IAA inoculations. As expected, branching was significantly reduced upon addition of TIBA (Fig. 25 C). Efforts to test the hyphal branching effect of D'orenone, a precursor of trisporic acids, which was observed to inhibit plant root hair tip growth by interfering with PIN2-mediated intercellular auxin transport and signaling (Schlicht *et al*., 2008), resulted in branching pattern that was not significantly different from control mycelium (data not shown).

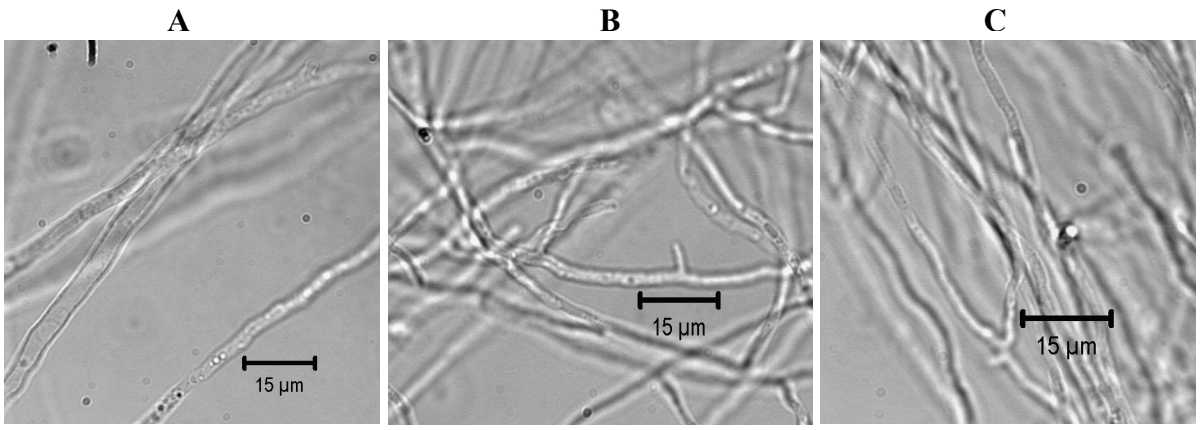


Fig. 25. Effect of IAA on *T. vaccinum* hyphal branching. 0.5 mM IAA, exogenously applied to fungal cultures, significantly increased fungal hyphal branching (B) compared to less or no branching observed in control (A). Co-inoculation of IAA with TIBA significantly reversed this phenotype (C).

3.3.2. Effect of IAA on *T. vaccinum* fungal growth

To test the growth response of the ectomycorrhizal fungus *T. vaccinum* to IAA, 100 µM of the phytohormone were exogenously applied to solid medium and growth quantified by measuring colony diameter. The growth measurements indicated that, although inoculation with IAA resulted in colonies larger than uninoculated controls, the difference was not statistically ($P<0.05$) significant (Fig. 26). However, application of 10 µM TIBA, the polar auxin transport inhibitor, either alone or together with 100 µM IAA, drastically reduced fungal growth (Fig. 26 and 27) clearly indicating that IAA is involved in *T. vaccinum* growth. In fact, the same observations were made when the experiment was carried out using 0.5 mM tryptophan as the IAA source, instead of pure synthetic IAA (data not shown).

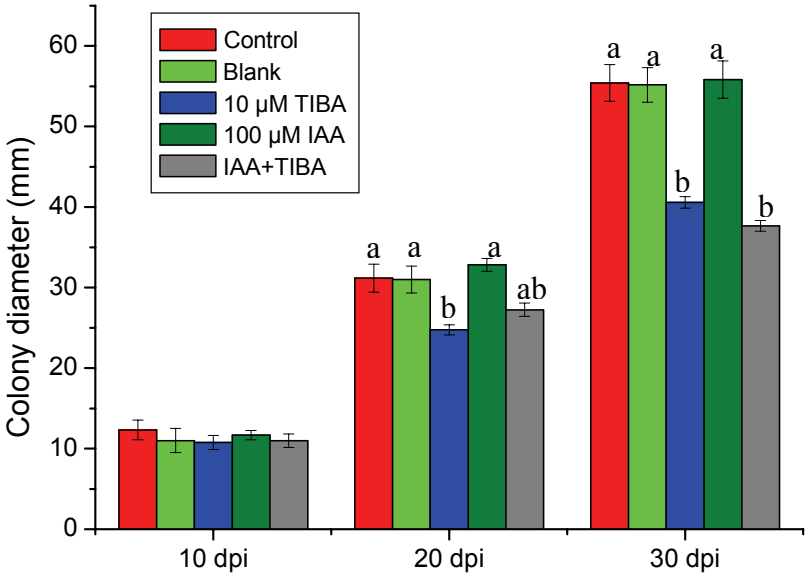


Fig. 26. Real time quantification of the effect of IAA on *T. vaccinum* growth. IAA and/or its polar transport inhibitor, TIBA, were exogenously applied to fungal MMNb culture plates. Fungal growth was quantified by measuring colony diameter. Bars denote standard error, and letters show significantly different treatments.

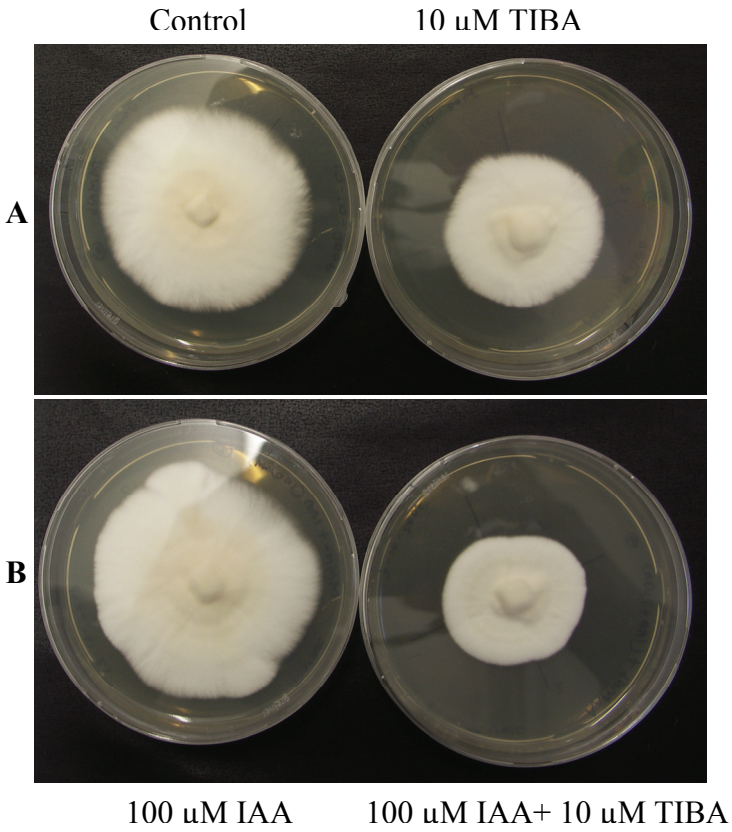


Fig. 27. Radial colony growth of *T. vaccinum* as influenced by IAA. IAA and/or its polar transport inhibitor, TIBA, were exogenously applied to MMNb culture plates. A: Comparison of control to TIBA; B: Effect of co-inoculating IAA with TIBA as compared to IAA a lone. 30 days old plates are shown.

3.4. Biosynthesis of indole-3-acetic acid in *T. vaccinum*

To validate the observation that *T. vaccinum* is able to produce IAA both with and without precursors, as shown by Salkowski assay, IAA in fungal culture filtrates was quantified by Gas Chromatography-Mass Spectrometry (GC-MS). Although IAA was detected in cultures without precursor supplements, indicating ability of the fungus to produce IAA on its own, the amounts were too low to be quantified since they were outside the calibration range established for the measurements (Fig. 28 and 29). However, the GC-MS results unequivocally demonstrated the ability of *T. vaccinum* to produce IAA in the presence of its precursors.

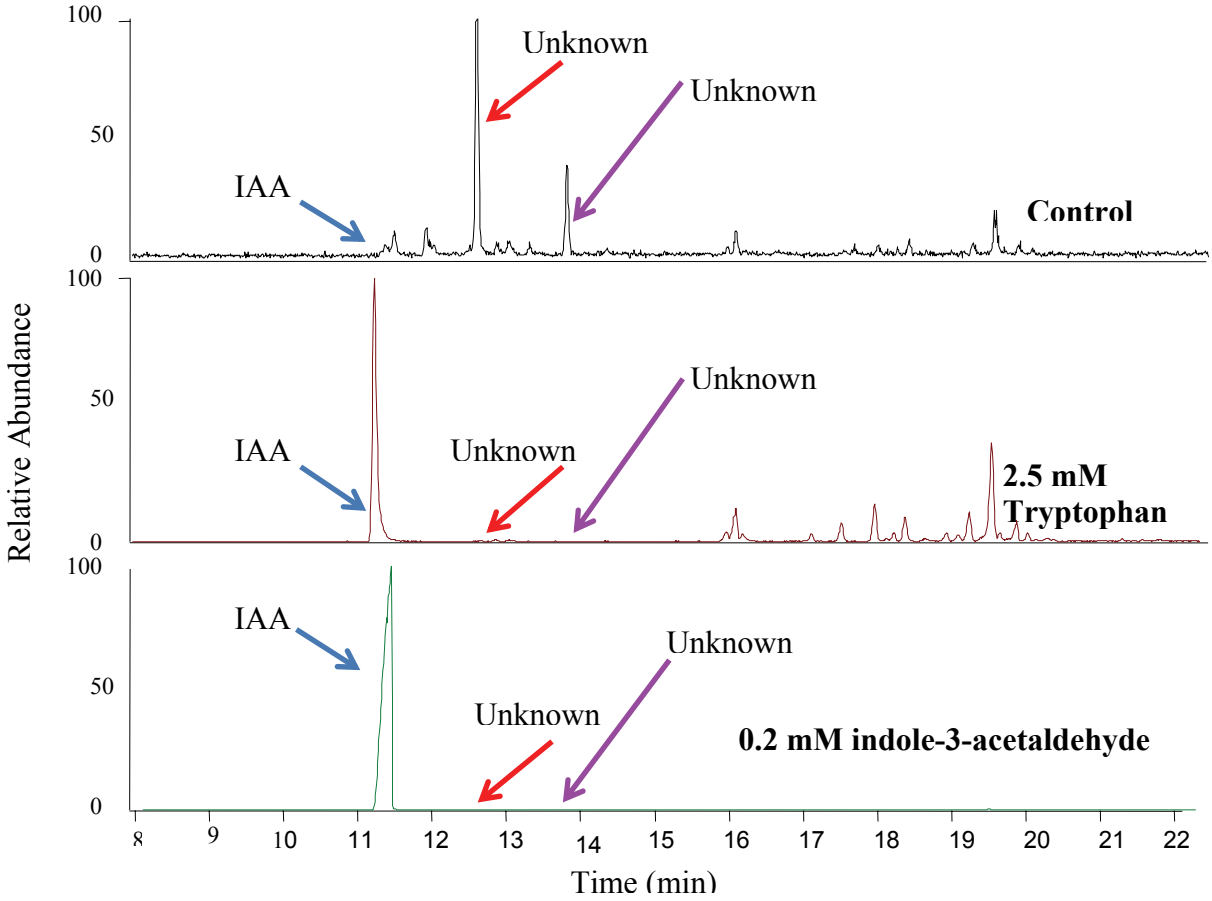


Fig. 28. Representative chromatograms of IAA purified from *T. vaccinum* culture filtrates containing the IAA precursors tryptophan and indole-3-acetaldehyde as compared to control cultures without precursor amendments (in bold). IAA was purified from 4 week-old cultures and analyzed by GC-MS. IAA peaks were obtained by tracing ($^{13}C_6$)-IAA.

Inoculation of fungal cultures with 0.2 mM IAAld produced high amounts of IAA, an unsurprising observation since it is expected to be an immediate precursor of the phytohormone (Table 9). The possibility of IAA being produced from IAAld by non-

enzymatic oxidation of IAAld was tested by inoculating the medium, without the fungus, with 0. 2 mM IAAld under the same growth conditions. Measurements using GC-MS did not detect quantifiable IAA, indicating that the oxidation of IAAld to IAA earlier observed is purely enzymatic, with IAAld aldehyde dehydrogenase as the most probable enzyme candidate.

Exogenous application of tryptophan to fungal cultures produced significant amounts of IAA even at 0.5 mM, the lowest concentration tested (Fig. 28 and Table 9). The IAA amounts were, however, much less than those in cultures supplemented with 0.2 mM IAAld, an expected observation since a small fraction of tryptophan would be expected to be finally converted to IAA, the rest going into the production of other metabolic compounds. Also, an attempt was made to test the transient production of IAA from tryptophan by measuring IAA, in real time, every week until the normal 4 week fungal growth period. The results indicated that IAA production is significant even during the first week of incubation, but steadily increases and reaches a maximum in the 4^{th} week (data not shown). Since all IAA measurements were done on 4 week-old fungal cultures, this test rules out the possibility of underestimating the IAA production potential of *T. vaccinum*. Furthermore, quantification of IAA in culture filtrates containing D'orenone revealed accumulation of IAA in the cultures, which was concentration-dependent (Fig. 29 and Table 9). However, visual observations indicated that D'orenone significantly reduced fungal growth (Fig. 30). Important to note in the quantification of IAA in fungal cultures (Table 9) is the fact that there was high variation between replicates.

To unequivocally prove the biosynthesis of IAA from tryptophan, ^{13}C-labeled tryptophan was fed and ^{13}C-labeled IAA measured. The production of labeled IAA could be shown, which, as earlier observed with unlabeled tryptophan, increased over time (Fig. 31). Interestingly, the labeled tryptophan feeding seemed to stabilize the variations in IAA amounts between replicates, which were earlier observed in unlabeled tryptophan feeding experiments, probably due to increased reproducibility.

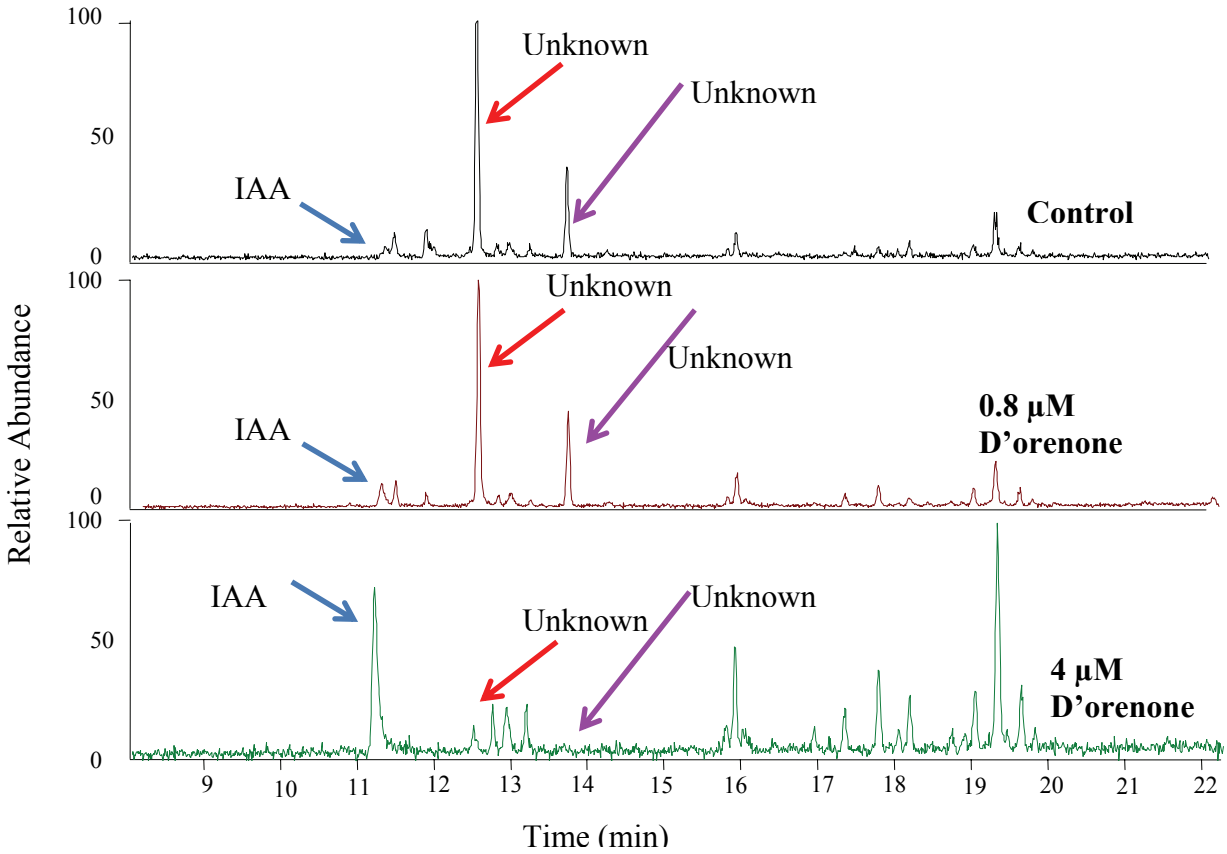
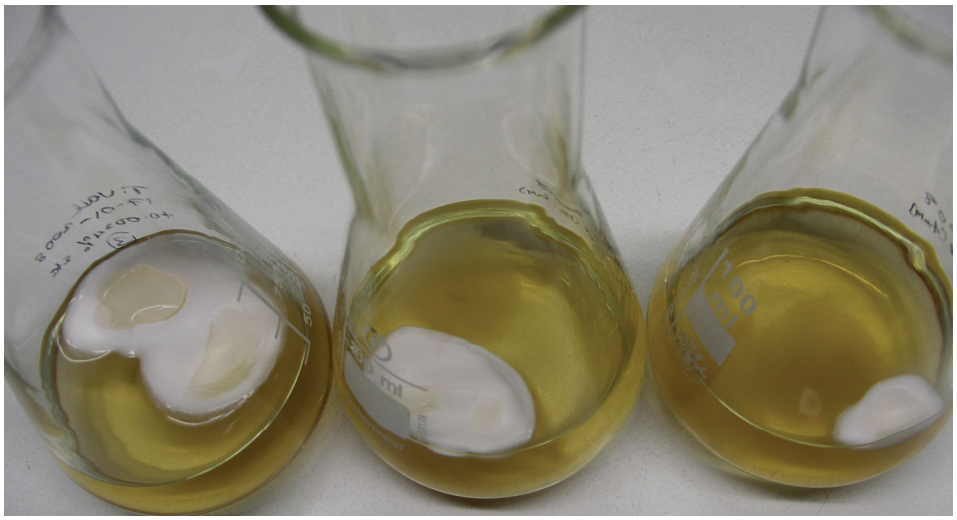


Fig. 29. Chromatograms of IAA purified from *T. vaccinum* culture filtrates containing polar auxin transport and signaling inhibitor, D'orenone, as compared to control cultures without the compound (in bold). IAA was purified from 4 week-old cultures and analyzed by GC-MS. IAA peaks were obtained by tracing ($^{13}C_6$)-IAA.

Table 9. Production of IAA by *T. vaccinum*, as quantified by GC-MS measurements

Treatment	IAA concentration (µM)
0.8 µM D'orenone	0.06
4 µM D'orenone	0.17
0.5 mM tryptophan	1.20
2.5 mM tryptophan	3.99
0.2 mM IAAld	140.39

A close observation of chromatograms generated during IAA measurements by GC-MS revealed two unknown compounds with retention times of about 12.5 and 13.5 min (Fig. 28 and 29). Interestingly, addition of IAA precursors, tryptophan and IAAld, as well as its transport and signaling inhibitor, D'orenone, downregulated the production of the compounds. Although the chemical structures of these compounds were not determined, MS results show that both of them possess an indole ring.



A B C

Fig. 30. Representative 4 week-old cultures of *T. vaccinum* showing biomass reduction in the cultures supplemented with D'orenone. Exogenous application of 0.8 µM (B) and 4 µM (C) D'orenone significantly decreased fungal colony size as compared to the control (A) without D'orenone application.

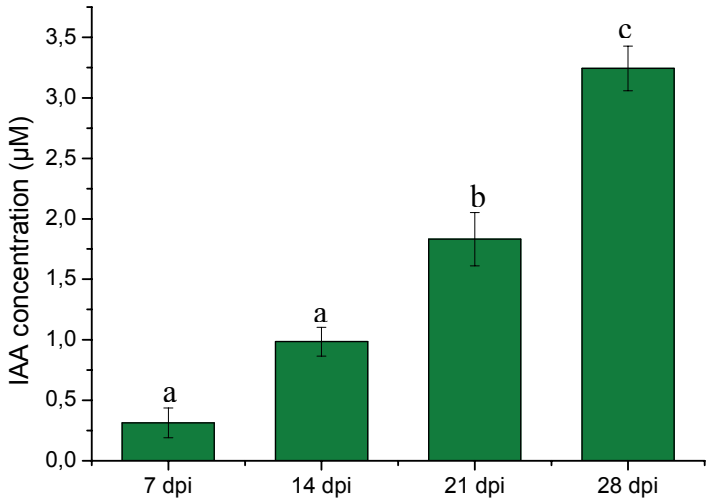


Fig. 31. Real time quantification of IAA from *T. vaccinum* cultures supplemented with labeled tryptophan. IAA was purified from 1, 2, 3 and 4 week-old cultures and analyzed by GC-MS. IAA peaks were obtained by tracing ($^{13}C_6$)IAA. The IAA amounts shown represent only IAA from labeled tryptophan. Bars denote standard error, and letters show significantly different IAA amounts.

3.5. Role of indole-3-acetic acid in ectomycorrhiza formation

3.5.1. Influence of IAA on *T. vaccinum*-spruce ectomycorrhizal development

The effect of IAA on establishing ectomycorrhizal features was investigated in co-cultures. In order to minimize the time to establish contact and therefore to increase chances of

mycorrhiza establishment, MMNa-medium was supplemented with 2 g/l glucose. Microscopic observations over time indicated that the mycorrhizal cultures established the same level of fungal mantle development, which was higher than that without glucose, regardless of whether they were treated with IAA or not. However, the observations show good Hartig' net establishment in cultures supplemented with 100 µM IAA compared to controls without IAA in which there was hardly any Hartig' net formation (Fig. 32). The effects of TIBA, an IAA inhibitor, could not be quantified since the controls without the compound also hardly established a Hartig' net (Fig. 32). Despite the fact that the fungus established contact with all the roots, it was only able to easily penetrate them in the presence of IAA, as shown by DAPI-stained fungal nuclei between cortical cells (Fig. 32C) and highly developed Hartig' net (Fig. 32F).

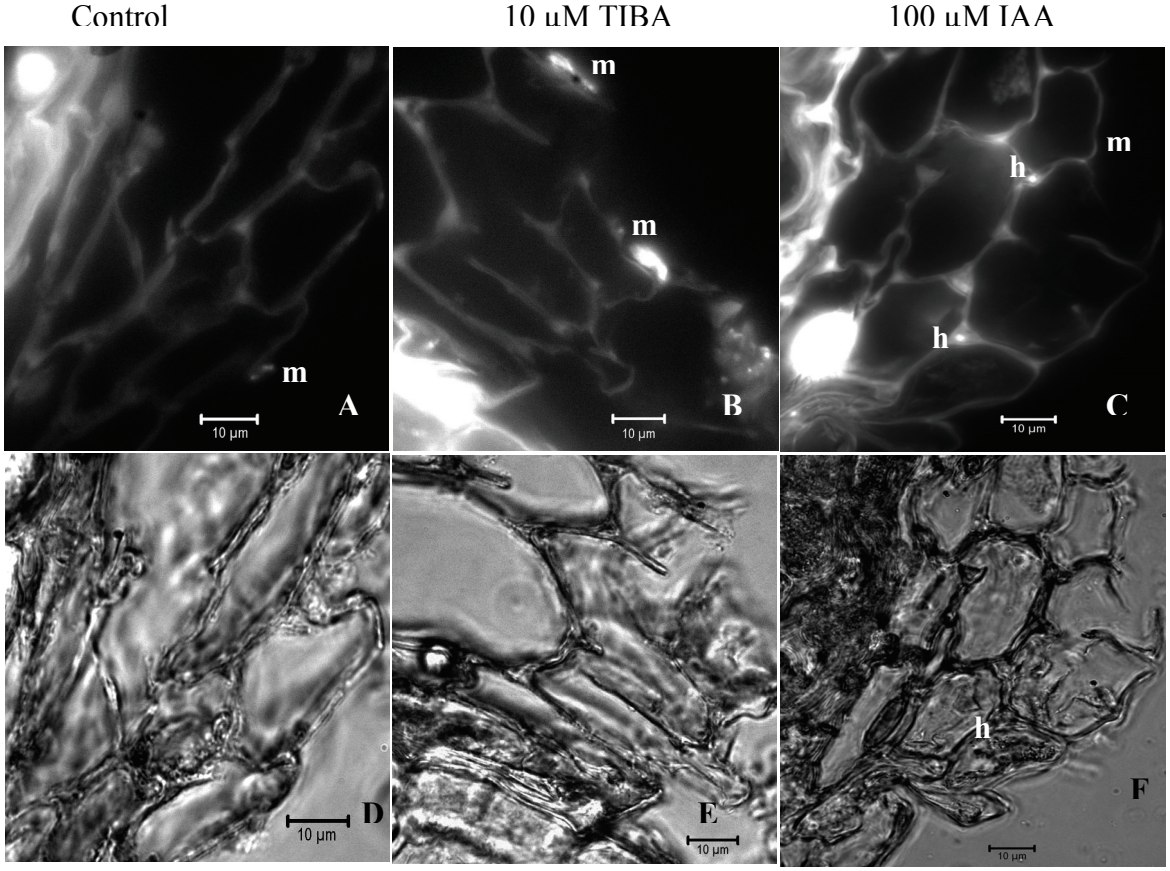


Fig. 32. *T. vaccinum*-spruce ectomycorrhizal development, as influenced by IAA after about 2 months of co-cultivation. Cryosections of mycorrhized seedling roots were obtained for visualization of fungal nuclei, after DAPI staining. Fluorescence microscopic visualization of stained nuclei (A, B and C), together with the corresponding bright field images (D, E and F) showed considerably high Hartig' net (h) development between cortical cells as a consequence of exogenous application of 100 µM IAA (C and F). Hartig' net was neither observed in mycorrhiza without IAA application (A and D) nor in mycorrhiza with exogenous application of 10 µM TIBA (B and E). However, the fungal mantle (m) was observed in all treatments.

3.5.2. Effect of fungal and IAA inoculation on plant growth and development

Plant growth promotion by ectomycorrhiza was tested *in vitro*. Spruce seedling lateral root and leaf needle development were quantified in mycorrhizal cultures supplemented with IAA or TIBA. The results indicate that fungal inoculation, either on medium with (Fig. 34) or without (Fig. 33A) glucose, repressed lateral root development up to 2.8 fold (Fig. 34, 28 dpi). The repression of lateral root development was, however, reversed by exogenous application of 100 µM IAA (Fig. 33A and 34). Furthermore, inoculation of *T. vaccinum* did not increase seedling shoot development, and exogenous application of 100 µM IAA never compensated for this shortfall (Fig. 33B). In fact, like the observed effect on lateral root development, fungal inoculation slightly slowed down shoot development. The results also indicate that IAA inoculation reduces shoot development, at least under the artificial co-inoculation conditions.

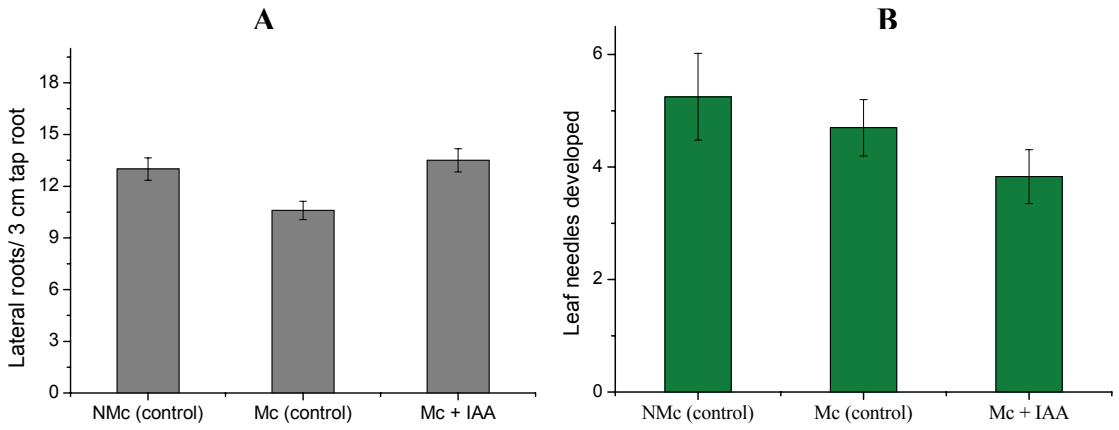


Fig. 33. Quantification of plant growth parameters in *T. vaccinum*-spruce ectomycorrhizal cultures established on MMNa-medium without glucose supplements. Spruce seedling lateral root (A) and leaf needle (B) development was quantified in 42 day-old mycorrhiza cultures. NMc, non-mycorrhized; Mc, mycorrhized. For IAA treatment, 100 µM of the phytohormone were exogenously applied to the cultures. Bars denote standard error.

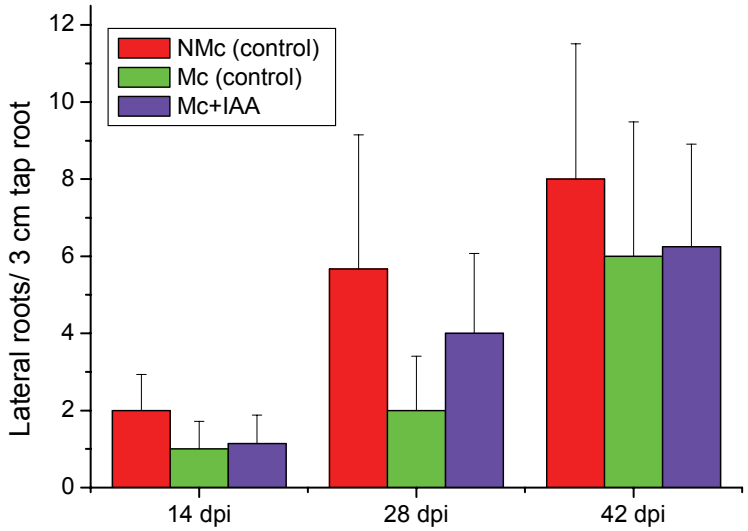


Fig. 34. Real time quantification of lateral root development on *T. vaccinum*-spruce ectomycorrhizal cultures established on MMNa-medium supplemented with 2g/l glucose. For IAA treatment, 100 µM of the phytohormone were exogenously applied to the cultures. NMc, non-mycorrhized; Mc, mycorrhized. Bars denote standard error.

4. DISCUSSION

4.1. Characterization of an ectomycorrhizal fungal aldehyde dehydrogenase

4.1.1. The structure and phylogeny of *ald1*

The molecular investigation of mechanisms underlying ectomycorrhiza formation and functioning has received less attention, yet it is fundamental to understanding this symbiotic association. Here, a full-length aldehyde dehydrogenase gene (*ald1*) from an ectomycorrhizal agaricomycete fungus *Tricholoma vaccinum* was characterized. The gene had earlier been shown to be differentially expressed in *T. vaccinum*-spruce ectomycorrhiza (Krause, 2005; Krause and Kothe, 2006) and therefore was expected to play a crucial role in the association. With 16 introns, *ald1* is an intron-rich gene, encoding the ALDH protein Ald1 of 502 amino acids. The molecular mass was calculated to be 53.5 kDa, with an isoelectric point of 6.28. The molecular mass was experimentally verified by heterologous expression of *ald1* cDNA in *E. coli*, followed by Western blot analysis. A second protein band observed on the Western blot (Fig. 11) could have resulted from protein degradation. As suggested by Pflug *et al* (2007), the low protein yield could be associated with the slowed transcription and translation machinery of the *E.coli* cells used in pET-system at the low temperature of 18ºC, at which the cultures were incubated.

The heterologous *ald1* expression confirmed the integrity of the cloned gene. As ALDH activity could be shown in the overexpressing *E. coli* cells, the cDNA cloned was functional. Both NAD^+ and $NADP^+$ were accepted as cofactors, agreeing with the potential binding sites for both cofactors identified in the Ald1 sequence. To the best of our knowledge, this is the first *ald* gene to be characterized in mycorrhizal fungi.

The availability of a sequenced genome of another ectomycorrhizal agaricomycete fungus, *Laccaria bicolor*, greatly facilitated the analysis of Ald1 structure and phylogeny. An interesting feature about ALDHs is a large extent of homology of these enzymes across all eukaryotic phyla. The alignment of the Ald1 amino acid sequence with other fungal ALDHs revealed similarities of up to 74%, with *L. bicolor* showing the highest amino acid similarity. ALDHs, which can oxidize different aldehydes, are grouped in an enzyme superfamily sharing 10 highly conserved motifs, which were suggested to cluster near the active site of ALDH enzymes (Perozich *et al*., 1999), and residues as reported in Hempel *et al*. (1993) and Perozich *et al.* (1999). Apparently, this might help to deduce the potential biological function

of Ald1. However, with the exception of *Ustilago maydis*, none of the related enzymes have been functionally characterized.

At the time Perozich *et al.* (1999) reconstructed ALDH phylogeny by using the 145 known amino acid sequences available by then, the fungal ALDH family was poorly represented. We have re-investigated the evolutionary relationships of fungal ALDHs, since now a considerable number of fungal genomes, including agaricomycetes previously not represented, have been sequenced. Because of this wider fungal ALDH resource base, we used a total of 53 specific fungal ALDHs, representing all major phyla in the kingdom of fungi, compared to Perozich *et al*. (1999) where only 9 specific fungal ALDHs were used. Database mining, using Ald1 as an amino acid sequence search querry, indicated that most fungal ALDHs have multiple paralogs, suggesting possible duplication events during the course of their evolution. With this in mind, a phylogenetic reconstruction of fungal ALDH revealed that fungal ALDHs diverged in accordance with the conventional fungal classification based on morphological and molecular characteristics, as evidenced by clustering of ALDHs in distinct taxonomic groups, with chytridiomycota being basal to all other fungal groups (Blackwell *et al*., 2009). However, the occurrence of two distantly related groups of each taxon, with some fungi possessing multiple paralogs, implies early duplication and divergency of this enzyme family. Indeed, a closer look revealed two possible major duplication events during the course of evolution, the first duplication happening after the split of chytridiomycetes and another, more recent one, within the agaricomycetes (Fig. 10). These possible duplication events are also supported by the fact that higher fungi posses higher numbers of paralogs compared to lower fungi. Furthermore, multiple fungal ALDH sequence alignments had indicated that the sequence of *Coprinopsis cinerea* 4, which clusters in the agaricomycetes clade (basidiomycota I group) (Fig. 10), had undergone deletion events, including essential conserved motifs. This would suggest that this paralog is non-functional. Another surprising observation is the fact that the phylogram depicts ascomycetes being basal to zygomycetes. However, this could be due to the effect of long branch attraction (reviewed in Bergsten, 2005). Nonetheless, the fact that phylogenetic analyses using Treefinder, RAxML and PAUP generated the same topologies with that of MrBayes unequivocally demonstrated the reproducibility of our phylogenetic investigation.

Although *T. vaccinum* has received little research attention so far, and its genome has not been sequenced, a total of three *ald* paralogs, including the now fully sequenced *ald1*, have

been reported (Krause, 2005; Asiimwe *et al*., 2010). Obviously, the total number of paralogs in the genome of this ectomycorrhizal agaricomycete fungus can not be deduced until the genome is sequenced. However, since all basidiomycetes with sequenced genomes, including close relatives of *T. vaccinum*, have paralogs that cluster in basidiomycota 2 group (Fig. 10), we predict at least one additional *T. vaccinum* paralog in this group. Nonetheless, the identified paralogs are likely to play roles in the symbiotic association between the fungus and its compatible host plant spruce (*Picea abies*), since their "parent" fragment was differentially expressed in the ectomycorrhiza (Krause and Kothe, 2006). Until the biological functions of the paralogs are investigated in detail, it will remain a matter of speculation that the functions of *ald2* and *ald3* are not so different from those of *ald1* considering the close clustering in the phylogram. Assuming the current status of fungal ALDH evolution, *T. vaccinum* ALDH amino acid sequence is suggested to have diverged relatively recent, compared to other agaricomycetes.

4.1.2. Regulation of *ald1* gene expression

Analysis of the promoter region of *ald1* revealed the presence of five putative Stress Response Elements (STREs). These elements have been detected, and suggested, to be involved in induction of stress-inducible genes (Martinez-Pastor *et al*., 1996; Görner *et al*., 1998; Moskvina *et al*., 1999; Zähringer *et al*., 2000). The presence of such elements is tempting to suggest that *ald1* is induced under stress conditions. However, reports of STRE elements in promoter regions of fungal *ald* genes are rare, which is also the case with other *ald* genes across all phyla, yet this is one of the gene superfamilies that have been widely studied. All reports on *ald*, nonetheless, agree that these genes are stress-inducible, although some level of constitutive expression also exists, depending on the ALDH class, and conditions prevailing. The presence of the putative transcriptional control sequences, the "GC" boxes (consensus sequence GGGCGG), which bind the transcription factor Sp1, common in constitutively expressed genes (Dynan and Tjian, 1985), in the promoter region of *ald1*, hints on the fact that it may also show a low constitutive, and stress-inducible expression pattern.

Alcohol- and aldehyde-related stress effects may be different from those of the stress factors like heat, osmosis, low pH and nutrient starvation, mentioned in the studies demonstrating the function of STREs. However, induction by various aldehydes and ethanol could be expected. Using real time RT-PCR, it was established that 0.1 mM indole-3-acetaldehyde and

benzaldehyde significantly induced *ald1* expression by 3 fold and 4 fold, respectively. A relatively low concentration of ethanol (0.01%) increased *ald1* expression by 4 fold while 0.1% ethanol only led to 1 fold increase in transcript accumulation. This may be attributed to ethanol toxicity, which is, in fact, supported by the generally observed reduced fungal biomass in cultures containing 0.1% ethanol. These results support the earlier findings in the PhD work of Krause (2005), which were carried out by using competitive RT-PCR. The induction of *ald1* transcription only at low levels of ethanol probably implies mediation of *ald1* in mitigating stress effects resulting from ethanol, only at low concentrations that may be physiologically relevant.

The level of induction, however, was lower than the 50 fold increase observed by Krause (2005). A number of reasons could have brought about this discrepancy. Firstly, the fungal medium in the current study contained 20 g/l of malt extract, instead of 5 g/l used by Krause (2005). This could have resulted in metabolic accumulation of carbon compounds, including, but not limited to, glucose, in the fungal cultures, which would have then repressed *ald1* expression. Glucose is considered as a strong repressor of the ethanol regulon in *A. nidulans*, of which aldehyde dehydrogenase gene *aldA* is part, although other carbon compounds like fructose and lactose were also identified as weak repressors (Fillinger *et al*., 1995). The ethanol utilization (*alc*) pathway in *A. nidulans* is a model system for studying gene regulation (Felenbok *et al*., 2001), implying that alcohol and aldehyde metabolism of *T. vaccinum* is most likely regulated similarly. Secondary, the *T. vaccinum* strain used had undergone a prolonged sub-culturing regime from the time it was last used in *ald* expression studies by Krause (2005), without contact with the host plant. Since *ald1* was differentially expressed in ectomycorrhiza (Krause and Kothe, 2006), implying a probable requirement for plant signals, the sub-culturing of the fungus on rich medium could have led to a relaxed *ald* induction system resulting in weaker transcript accumulation. Thirdly, the inaccuracy of competitive RT-PCR, just like any other conventional RT-PCR-based procedures in quantifying gene transcript accumulation (Souaze *et al*., 1996; Bustin, 2000), could have inflated the estimated gene transcript to higher levels. On the other hand, after reviewing all mRNA quantification methods, Bustin (2000) concluded that real time RT-PCR, which was used in the present study, must be a method of choice for any experiments requiring sensitive, specific and reproducible quantification of mRNA. Nonetheless, these two studies agree on the fact that ethanol, at low levels of concentrations, induce *ald1* gene expression.

Ethanol, together with other alcohols, was also observed to induce the transcription of *aldA* from the model fungus *A. nidulans* (Fillinger *et al*., 1995; Flipphi *et al*., 2001). However, Flipphi *et al*. (2001) clearly indicated that the real physiological inducers of the *aldA* gene expression, by using alcohols as the co-inducing compounds, are the corresponding aldehydes produced by oxidation of the respective alcohols. Therefore, the induction of *ald1* transcription by ethanol would indirectly implicate acetaldehyde as the real physiological inducer. Unfortunately, this could not be confirmed by using acetaldehyde as an *ald1* gene inducer due to its high volatility at room temperature.

ALDHs are generally regarded as detoxification enzymes (Jacoby and Ziegler, 1990), largely because they are involved in oxidation of various aldehydes to non-toxic, usable carbohydrates. The fact that *ald1* was induced by both aliphatic and aromatic aldehydes (Krause, 2005), which has been confirmed in the present study by using real time RT-PCR, where for example the aromatic aldehydes indole-3-acetaldehyde and benzaldehyde strongly induced the gene expression, may imply that either the gene product has a wide substrate specificity or is simply induced as a consequence of the general stress effects resulting from these aldehydes. It is tempting, therefore, to speculate that *ald1* gene may be involved in the production of the phytohormone indole-3-acetic acid (IAA) since ALDH has been reported to catalyze the oxidation of indole-3-acetaldehyde to IAA (Cooney and Nonhebel, 1989; Basse *et al*., 1996; Tam and Normanly, 1998; Fedorova *et al*., 2005; Spaepen *et al*., 2007; Reineke *et al*., 2008). In fact, this argument is further supported by the fact that Ald1 has an amino acid identity of 58% with *U. maydis* indole-3-acetaldehyde dehydrogenase, iad1, which is involved in production of IAA (Basse *et al*., 1996).

The results, taken together, suggest that *T. vaccinum ald1* is likely to play two important roles in ectomycorrhiza *in vivo*. Firstly, it may be involved in detoxification of alcohols and aldehydes that tend to accumulate in ectomycorrhizal habitats, especially under conditions of flooding and water logging. Secondary, the possible involvement of *ald1* in IAA production would facilitate mycorrhiza establishement and maintanance, as reviewed in Podila (2002).

4.1.3. The function of *ald1* in *T. vaccinum*

In order to understand the biological function of *ald1* in *T. vaccinum*, the gene was overexpressed in the fungus by using *A. tumefaciens*-mediated transformation (ATMT). Hygromycin resistant fungal transformants haboring *ald1*-overexpression vectors were screened on selection plates, and confirmed by PCR. Furthermore, tests confirmed that the

transgene was stably integrated into *T. vaccinum* genome, which is in agreement with the findings of Schlunk (personal communication) who also observed the stability of *hgh* in ATMT of this fungus using the plasmid pBGgHg (Chen *et al*., 2000), which forms the backbone of the plasmids used here. Schlunk, using the same plasmid backbone, found predominantly single copy integrations (Schlunk, personal communication), which has also been reported elsewhere (reviewed in Meyer, 2008).

A PCR targeting the *egfp* gene on one of the transforming plasmids had indicated that this gene was successfully integrated into the genome of *T. vaccinum* (Fig. 18 B). In an effort to investigate whether the gene was functionally expressed in *T. vaccinum*, eGFP-associated green fluorescence in detached mycelium of transformants was monitored by confocal laser-scanning microscopy. Preliminary results revealed cytoplasmic eGFP-associated green fluorescence in the transformants with some accumulation in specific regions, probably nuclei (Fig. 20), which is typical for all eukaryotic organisms (Fabre and Hurt, 1994; Müller *et al*., 2006). Unfortunately, this was not reproducible in subsequent fungal sub-cultures. In these cultures, the eGFP-associated green fluorescence was drastically reduced to background fluorescence comparable to the wild type. This could have been either due to a possible difficulty in the *egfp* transgene expression or its instability upon sub-culturing of the fungal transformants on non-selective plates. Although reporter GFP green fluoresecnce has been reportedly detected in a few agaricomycete fungal transformants (Burns *et al*., 2005; Rodríguez-Tovar *et al*., 2005; Müller *et al*., 2006), other authors had reported difficulties in *egfp* gene expression. For example, Chen *et al*. (2000), Hanif *et al*. (2002) and Combier *et al*. (2003) were unable to observe eGFP fluorescence in the transformants of agaricomycetes *A. bisporus*, *Suillus bovinus* and *Hebeloma cylindrosporum*, respectively, transformed with pBGgHg, which formed the backbone of the current transforming plasmids. However, like in the present study, the authors were able to prove, by Southern blot analysis, PCR and mitotic stability tests, that *egfp* was successfully integrated into the fungal genomes. Other cases where GFP expression has been problematic, which was actually attributed to codon preference, are the yeast fungus *C. albicans* (Cormack *et al*., 1997) and the basidiomycete fungus *U. maydis* (Spelling *et al*., 1996), not to mention other organisms. In fact, even in the most of the successful stories in GFP expression, it was shown that an intron fusion to *gfp* gene was needed before significant levels of protein expression were seen. However, the recent reports of eGFP expression without the requirement of an intron (Rodríguez-Tovar *et al*., 2005; Müller *et al*., 2006) suggests the existence of other impedements to significant

eGFP expression. These were observed to include preferential methylation, and inactivation of gene expression by AT-rich sequences like *egfp*, among others (Burns *et al*., 2005). Nevertheless, the expression of eGFP in *T. vaccinum*, albeit being unstable, demonstrates the feasibility of using *egfp* as a reporter gene in this ectomycorrhizal fungus. The enhancement of gene expression will, however, be necessary before full exploitation of GFP as a reporter in this fungus is possible.

Although, over the past decade, ATMT has been developed for a number of filamentous fungi, the validation of the method in functional gene analysis is rare. This is, therefore, one of the few pioneering studies that are, of recent, taking a step further from describing transformation methods to utilizing them for the ultimate purpose of analyzing gene functions. For ectomycorrhizal fungi, this is the second demonstration of using ATMT in investigating a gene function. Investigation of gene function using agrotransformation in ectomycorrhizal fungi was first demonstrated by heterologously overexpressing a *Paxillus involutus* metallothionein in *Hebeloma cylindrosporum* (Bellion *et al*., 2007). In this study, the possible mitigation of ethanol- and aldehyde-related stress by *T. vaccinum* aldehyde dehydrogenase, Ald1, was functionally investigated in a fungal transformant overexpressing the enzyme. Real time experiments with 2% ethanol showed that the transformant tremendously reduced the ethanol stress, compared to the wildtype. These results clearly indicate that *ald1* mediates, at least partly, ethanol stress tolerance of the ectomycorrhizal fungus *T. vaccinum*. Since ALDH is the second enzyme in ethanol catabolism (Flipphi *et al*., 2001), Ald1 could have acted by oxidizing the rather toxic acetaldehyde, produced by an alcohol dehydrogenase-mediated oxidation of ethanol. This confirms that ethanol- and aldehyde-induced *ald1* gene expression is linked to ethanol detoxification, a function that is directly relevant in ectomycorrhizal habitats. Interestingly, concentrations of ethanol lower than 2% resulted in higher growth compared to controls without ethanol, a trend that was observed both in the transformant and wild type strains. This, unsurprisingly, suggests that, with the help of ADH and ALDH, the fungus is able to utilize ethanol as a carbon source for its energy requirements.

It has been shown, in separate experiments, that *ald1* gene is significantly induced by benzaldehyde, but does not necessarily alleviate benzaldehyde-related stress. Also, Ald1 enzyme activity assays indicated that benzaldehyde does not react with the enzyme. This implies that the aromatic aldehyde is not a substrate for Ald1, and that the induction of *ald1* gene expression earlier observed could, most likely, be due to a general stress stimulus acting

by a different pathway. However, we could show that aromatic substrates are not completely rejected. Indole-3-acetaldehyde induced gene expression and was accepted as a substrate in enzyme activity assays. This means that Ald1 may be, at least partly, involved in oxidation of indole-3-acetaldehyde to indole-3-acetic acid. Nevertheless, Ald1 exhibited a strong activity on the aliphatic aldehyde propionaldehyde, but with a 13 fold higher activity when using β-$NADP^+$ as a cofactor than with β-NAD^+. The strong preference of propionaldehyde and the predicted, and GFP-confirmed cytosolic localization is in agreement with previously studied substrate specificities of other cytosolic aldehyde dehydrogenases (Chen *et al*., 2008; Guru and Taranath-Shetty, 1990).

The fact that a low enzyme activity was also detected while using β-NAD^+ as a cofactor on propionaldehyde, and that another aliphatic aldehyde, butyraldehyde, was accepted only by using β-NAD^+ as a cofactor, shows that Ald1 is able to bind both cofactors with different affinities. This is, in fact, supported by the fact that both the signature sequences for NAD binding (GXTXXG; Lie *et al*., 1997) and the general glycine motif for NADP binding (GXGXXXG; Ferres *et al*., 1989; Jung and Lee, 2006) were present in Ald1. This may sound unusual for fungal ALDHs, which were suggested, by Perozich *et al*. (1999), to be NAD-dependent enzymes. The same authors, despite their acknowledgment of difficulty in assigning co-enzyme preferences to ALDHs, went ahead to suggest that with the exception of class 3 ALDHs, which can utilize NAD or NADP, other ALDHs can only utilize one of the cofactors. Obviously, it was too early to make such conclusions, especially in the case of fungal ALDHs, which were poorly represented at that time. Since then, several authors have revisited this topic, and what is clear now is the fact that some ALDHs have the ability to bind the two cofactors, albeit with different affinities. For example, Isobe *et al.* (2007) reported the isolation of an ALDH from *Rhodococcus sp*, which was able to utilize various aldehydes by using both β-$NADP^+$ and β-NAD^+ as cofactors, with the activity with β-$NADP^+$ being about 5% of that with β-NAD^+. Another intriguing observation is the fact that whereas Perozich *et al*. (1999) indicated that betaine ALDH prefers NAD as a cofactor, different authors like Velasco-García *et al*. (2000) and Mori *et al*. (2002), among others, showed that the enzyme can actually utilize both cofactors. This dual cofactor preference phenomenon of some ALDHs had been known even earlier since a cytosolic ALDH from a rat liver (Marselos and Lindahl, 1988) and later, a fungal ALDH from *Saccharomyces cerevisiae* (Wang *et al*., 1998) were observed to utilize both cofactors.

Assuming that some ALDHs are, indeed, able to utilize both cofactors, the question then arises as to what mechanisms could be employed by these highly versatile enzymes for this

purpose. It may be that, ultimately, the prevailing NAD/NADP ratio determines, at least partly, which cofactor the enzyme will more readily utilize, but the cofactor with less affinity would of course remain at a physiological disadvantage at all times. Also, Perozich *et al*. (2000) indicated that class 3 ALDH, a model ALDH for the dual factor preference, may shift the cofactor preference to either NAD or NADP, depending on the prevailing conditions. Since their study has not been extended to other ALDHs that have been shown to bind the two cofactors, this may be an alternative explanation for their proven dual cofactor binding.

4.2. Indole-3-acetic acid in ectomycorrhiza

4.2.1. Indole-3-acetic acid biosynthesis, and influence on *T. vaccinum* biology

As has been observed and discussed, we speculated that *T. vaccinum* Ald1 may be involved in IAA production. Cultures incubated with indole-3-acetaldehyde (IAAld), an IAA precursor, show more branches than controls without IAAld supplements, an observation that was also earlier hinted on by Krause (2005). This prompted us to speculate that the increased hyphal branching may be due to the influence of IAA produced from IAAld. If so, this would be the first study to demonstrate the effect of IAA on ectomycorrhizal fungal biology, a possible fundamental phenomenon that would help to explain the involvement of IAA in ectomycorrhiza, which has been controversially observed and discussed in literature (see Podila, 2002).

Subsequently, by using different concentrations of IAAld to test our hypothesis, we observed a significant increase in hyphal branching at concentrations above 0.05 mM IAAld, with extensive branching at 0.2 mM IAAld. Calorimetric quantification of IAA showed significantly higher amounts in cultures supplemented with IAAld, suggesting that, indeed, IAA produced from IAAld was responsible for the observed branching phenotype. Another IAA precursor, tryptophan, also led to hyperbranching, which correlated with IAA amounts in cultures supplemented with the precursor. Exogenous application of pure synthetic IAA, either alone or simultenously with its transport inhibitor, TIBA, indicate that IAA significantly increased branching, starting at a concentration of 0.1 mM, with extensive branching at 0.5 mM. This phenotype was reversed by TIBA. These observations, the first of their kind in ectomycorrhizal fungi, unambuigously confirmed our earlier speculation that IAA increases hyphal branching in this ectomycorrhizal fungus.

At the same time, IAA had a positive impact on growth of the fungus. Exogenous application of TIBA in cultures drastically reduced growth, indicating that IAA is needed for fungal

growth. This was subsequently confirmed by co-inoculation of IAA or tryptophan with TIBA. Since TIBA interferes with IAA transport, the effect of TIBA can be explained with endogenously produced IAA that has to be transported between hyphal compartments, and between cytosol and vacuole. Clearly, these experiments show that IAA is involved in the growth of *T. vaccinum*. Although this is the first report on the effect of IAA on growth of a basidiomycete fungus, Nasim *et al*. (2004) also observed that the phytohormone promotes growth of soil ascomycete fungi. Another interesting observation is that IAA supplements seemed to increase fungal growth, although this was found to be statistically not significant. This may imply that under these *in vitro* conditions, which are not likely to prevail *in vivo*, and given the nutritional status of the rich medium used, the fungus produces enough IAA for its metabolic activities hence not requiring supplements. Furthermore, the observed reduction of fungal growth in TIBA-inoculated cultures implies blocking of endogenous IAA polar transport, meaning that the inhibitor was transported across the fungal cell plasma membrane from culture medium to the cell, and probably from cell to cell. In earlier studies involving corn coleoptiles (Thomson *et al*., 1973), TIBA intercellular transport was suggested to be polar, probably *via* the same channels as auxin.

Taken together, these results clearly indicate that IAA influence on *T. vaccinum* biology is two fold: The phytohormone increases growth of the fungus and hyphal ramification. The observed phenotypes of IAA and its inhibitor TIBA, the first of their kind in basidiomycete ectomycorrhizal fungi, thus far, suggest a profound influence of the phytohormone on ectomycorrhiza development. The rationale behind this suggestion is the assumption that with IAA or its precursor availabilty, most likely tryptophan, in ectomycorrhizal habitats, the phytohormone would increase fungal biomass and hyphal ramification, which would in turn increase Hartig' net formation in intercellular spaces of cortical cells. The result would then be an increased speed and efficiency of ectomycorrhiza development.

Salkowski assay suggested that *T. vaccinum* is able to produce IAA both with and without its precursors. However, before meaningful conclusions could be drawn from this observation, it was necessary to validate the calorimetric test with a more sensitive and accurate method of IAA quantification. Thus, IAA in fungal culture filtrates was quantified by using GC-MS. Although IAA production in fungal cultures without precursor supplements was insignificant and not quantifiable, considerable IAA amounts were detected in cultures supplemented with IAAld and tryptophan. In fact, the fungus produced high amounts of IAA in cultures supplemented with 0.2 mM IAAld, which was confirmed to be enzyme-driven, estimated to

be 35 fold that recovered in cultures supplemented with 2.5 mM tryptophan. This was not surprising, since IAAld is immediate IAA precursor. The fact that the oxidation of IAAld to IAA, the last step in tryptophan-dependent IAA biosynthetic pathway (Woodward and Bartel, 2005), is an enzymatic reaction, is supported by earlier findings of Basse *et al*. (1996), and later Reineke *et al*. (2008), that in the basidiomycete smut fungus *Ustilago maydis*, this step is catalyzed by an indole-3-aldehyde dehydrogenase. Nonetheless, significantly high amounts of IAA were also produced, by the fungus, from tryptophan, even at 0.5 mM, the lowest concentration tested. The low yield of IAA in tryptophan-inoculated fungal cultures could be due to the fact that tryptophan is subjected to metabolic losses *en route* to IAA because, according to Woodward and Bartel (2005), it is further upstream in the IAA biosynthetic pathways.

Apart from IAA precursors, D'orenone was tested. This compound resulted in formation of IAA, albeit at low levels, which was concentration-dependent. D'orenone was observed to increase PIN2 protein abundance leading to increased auxin efflux from *Arabidopsis thaliana* root hair trichoblasts resulting in blocking of polarized tip growth (Schlicht *et al*., 2008). Whereas this is a metabolically relatively less studied compound, the increase in IAA amounts observed in *T. vaccinum* cultures could be a result of increased IAA transport from fungal cells to the cultures caused by upregulation of a fungal PIN transporter. If this was the case, then the fungal biomass would be reduced as IAA was shown to be essential for *T. vaccinum* growth. Indeed, this seems to be the case as shown in Fig. 30, where the fungal colony size was drastically reduced by D'orenone application. Alternatively, the results could also imply that D'orenone itself could be involved in diverting tryptophan from being used for other metabolic activities, like protein synthesis, to IAA biosynthesis. Of course this would also lead to slower fungal growth, again resulting in smaller colony size. During IAA quantification by GC-MS, two unknown compounds, which seemed to be metabolically antagonistic to IAA, were observed in chromatograms. Although the MS results showed that the compounds possess indole rings, and could, therefore, be tryptophan analogs, the full identification of these compounds would shed more light on their possible involvement in IAA biosynthesis in *T. vaccinum*.

Most studies investigating IAA production by microbes did not proove that the IAA reported is, indeed, from tryptophan or other IAA intermediates fed, since unlabeled compounds were used. Whereas the authors were quick to make conclusions about specific IAA biosynthetic pathways, it could well be that a fraction of IAA amounts they report may have been derived from non-enzymatic oxidation reactions or other yet unidentified sources, in which case the

reported pathways would be questionable. To proove, beyond any reasonable doubt, that the IAA production kinetics for *T. vaccinum* reported here is accurate, we fed the fungus with labeled tryptophan, and tracked the isotope in IAA. Indeed, this study confirmed the fact that *T. vaccinum* produces IAA from tryptophan.

Until today, surprisingly, no single IAA biosynthetic pathway has been fully elucidated. This is even worse for fungi, where such studies are rare. Nevertheless, there are a number of uncomplete tryptophan-dependent IAA biosynthetic pathways, indicated in Pollmann *et al*. (2009), based on convincing evidence. Critical evidence of the possible biosynthetic pathways that could be used by fungi comes from *U. maydis*. It has been proposed that IAA biosynthesis in this fungus proceeds *via* IPyr, IAAld, and TAM (Basse *et al*., 1996; Bölker *et al*., 2008; Reineke *et al*., 2008; Zuther *et al*., 2008), but IPyr and IAM pathways have been proposed for *Colletotrichum gloeosporioides* and *C. acutum* (Robinson *et al.*, 1998; Chung *et al*., 2003). Although different authors have demonstrated the ability of some ectomycorrhizal fungi to produce IAA *in vitro* (Karabaghli *et al*., 1998; Rudawska and Kieliszewska-Rokicka, 1997), no investigation on IAA biosynthetic pathways has been reported for these fungi. The fact that *T. vaccinum* produced IAA both from tryptophan and IAAld suggests that this fungus employs the tryptophan-IPyr-IAAld-IAA biosynthetic pathway (Fig. 5), which is in agreement with one of the pathways proposed for *U. maydis* (Basse *et al*., 1996; Bölker *et al*., 2008; Reineke *et al*., 2008; Zuther *et al*., 2008). Feeding experiments with labeled IPyr could be employed to prove that this ectomycorrhizal fungus produces IAA *via* the proposed biosynthetic pathway. Investigation of the IAA biosynthesis by feeding labeled tryptophan to identify IAA intermediates has been launched to close this knowledge gap.

The *in vitro* studies on *T. vaccinum* have shown that the fungus produces IAA, primarily from precursors. On the other hand, the phytohormone was shown to increase fungal growth and ramification. These observations are supportive for a role of IAA in enhancing ectomycorrhization efficiency. Thus, with tryptophan availability in root exudates, this fungus will produce IAA, which in turn increases mycorrhization speed and efficiency. Root exudates were reported to stimulate hyphal growth of ectomycorrhizal fungi (Tagu *et al*., 2002). Although tryptophan was not quantified in these studies, it is known to exist in root exudates, and therefore the observed fungal growth could, at least partly, be attributed to this IAA precursor, making IAA the actual growth stimulator. The question, of course, would now be how much tryptophan would be expected to be in plant root exudates *in vivo*. While it is not expected to be at the level of concentrations used in various experiments to test the ability

of ectomycorrhizal fungi to produce IAA, it could, nonetheless, be sufficient to trigger the increased biosynthesis of IAA by ectomycorrhizal fungi under symbiotic interaction. After all, our results, as well as those of other authors, show that tryptophan concentration does not necessarily have to be high for significant IAA production. In fact, since rich medium suppresses IAA production (reviewed in Gogala, 1991), *in vivo* phytohormone levels are expected to be higher than what was observed in *in vitro* studies, including the current one, which are characterized by high nutrient levels. This corresponds to Slankis' claim (Slankis, 1973) that the absence of mycorrhizae in rich soil can be explained by inhibition of auxin synthesis in fungi under such conditions.

4.2.2. Ectomycorrhiza development, as influenced by indole-3-acetic acid

The controversial topic about the involvement of IAA in ectomycorrhiza (Podila, 2002; Barker and Tagu, 2000) was revisited in this study. Since IAA was shown to increase the growth and hyphal ramification of the ectomycorrhizal fungus *T. vaccinum*, with a possible consequential enhancement of ectomycorrhiza formation, we aimed at confirming this hypothesis by investigating the effect of IAA on differentiation of ectomycorrhizal features in *T. vaccinum*-spruce ectomycorrhiza *in vitro*. Initial attempts to investigate this, using culture medium without glucose supplements, prooved futile because, as observed in the cultures, fungal growth and colonization was poor leading to almost no Hartig' net formation. Whereas Hartig' net formation has been reported in cultures without addition of glucose (Asiimwe *et al*., 2010; Krause and Kothe, 2006), consistent observations over time indicate that *T. vaccinum*, routinely cultured on rich medium containing 10 g/l glucose and 20g/l malt extract, had led to difficulties for the rich medium-conditioned fungus to sufficiently grow on minimal medium in co-culture with rather small spruce seedlings, which offer less carbohydrates. Also, prolonged cultivation of the fungus on rich medium may have led to a decline in mycorrhization ability.

Since Giovannetti e*t al*. (1996) stated that the initial stages of mycorrhizal colonization events are dependent on saprophytic fungal growth, the experiment was repeated with culture medium containing 2 g/l glucose to increase fungal growth and, therefore, facilitate establishment of fungal contact with roots. Thus, exogenous application of 100 µM pure synthetic IAA in mycorrhiza cultures led to a well established Hartig' net, compared to cultures without IAA supplements, in which there was hardily any Hartig' net formation. At the same time, microscopic monitoring revealed the same extent of fungal mantle development, regardless of whether cultures received IAA supplements or not. This is

supported by the observed promotion of ectomycorrhizal development by exogenously applied IAA in oak-*Piloderma croceum* ectomycorrhiza cultures (Herrmann *et al*., 2004).

The results imply that the fungus was able to penetrate spruce seedling roots largely in the presence of IAA, although the fungus-root contact (mantle) was the same across treatments. This further suggests that IAA could have acted as a signal in communication between the fungus and seedling roots to facilitate entry into the roots, and subsequently establish a Hartig' net. This hypothesis is not far from that of Podila (2002) who suggested that by acting as a signal, IAA could trigger the transcription of auxin responsive genes, which would in turn trigger a cascade of molecular events in plant roots leading to formation of mycorrhiza. Contrary to the observations on *Pinus densiflora*-*T. matsutake* ectomycorrhiza (Yamada *et al*., 1999) and ectomycorrhiza between *Populus tremula* and various *Paxillus* isolates (Langer *et al*., 2008), that glucose supplementation improved ectomycorrhiza development, even after initial contact was established, this effect was not observed in *T. vaccinum*-spruce ectomycorrhizal cultures. In essence, therefore, the results suggest that while glucose increases fungal growth and hence the contact with roots, an IAA signal may be needed for the fungal-plant interaction to differentiate ectomycorrhizal structures. Since IAA accumulation in the ectomycorrhiza cultures of these authors was not quantified, their observations may not entirely be attributed to glucose.

The results of the current study support the elegant findings of Gay *et al*. (1994) who used IAA-overproducing transformants of *H. cylindrosporum* to convincingly demonstrate that IAA is involved in Hartig' net formation, although the mechanism leading to this phenotype was not known. Also, Rudawska and Kielszewska-Rokicka (1997) indicated that *Paxillus involutus* strains that were characterized by high IAA synthesizing activity induced significantly higher numbers of mycorrhizae than strains with low activity. Although there are a number of other studies supporting the hypothesis that IAA is important in regulating ectomycorrhiza colonization, other authors (Horan, 1991; Wallander *et al*., 1992; Wallander *et al*., 1994; Hampp *et al*., 1996) contested the IAA involvement. In a major critic of Slankis' auxin theory (Slankis, 1973), Wallander *et al*. (1992, 1994), based their suggestions on the fact that they never observed significantly high IAA amounts in mycorrhized compared to non-mycorrhized roots. However, they acknowledge difficulties in accurately quantifying IAA in plant roots. Thus, considering the existence of a multitude of convincing observations that support the auxin theory, and based on the current study, it is proposed that IAA is involved in regulating ectomycorrhiza formation.

Quantification of plant growth and development parameters in *T. vaccinum*-spruce ectomycorrhiza showed that inoculation of *T. vaccinum* repressed lateral root development. This repression was reversed by exogenous application of 100 µM IAA. Furthermore, fungal inoculation did not increase spruce seedling shoot development. Therefore, contrary to the current theory that mycorrhization increases plant growth and development, no evidence for plant growth promotion was observed in the current study under *in vitro* conditions. The results are supported by the observation that inoculation of *Piloderma croceum* onto oak microcuttings had no effect on the branching index of the first order roots (Herrmann *et al*., 2004). Contrary to these findings, in other earlier studies, inoculation of spruce seedlings with *Laccaria bicolor* increased lateral root development (Rincón *et al*., 2003; Karabaghli-Degron *et al*., 1998). Also, Smith and Read (1997) reported plant growth promotion by ectomycorrhiza in general. Karabaghli-Degron *et al*. (1998) could not rule out the fact that *L. bicolor* was able to increase lateral root development of spruce seedlings because of its ability to produce IAA *in vitro*. Therefore, the observed failure of *T. vaccinum* to promote spruce lateral root development and leaf needle development could be due to the fact that it does not produce quantifiable IAA without precursors *in vitro*. In fact, an elegant study by Scagel and Linderman (1998) showed that ectomycorrhizal fungi characterized by high IAA-producing capacity *in vitro* stimulated higher plant growth and development in seedlings transplanted to a forest site than those with low capacity. This, therefore, argues against the idea of a general effect of ectomycorrhizal fungi on plant growth and development promotion, and affirms the fact that the effects are likely to vary with different mycobiont-plant associations and with altering abiotic factors.

On the other hand, the promotion of lateral root development by IAA, which was also shown here, is now a widely accepted phenomenon. Also, like in the current study, Karabaghli-Degron *et al*. (1998) observed repression of spruce seedling needle development by IAA while Rincón *et al.* (2003) observed that the phytohormone does not have any effect on spruce seedling shoot growth and plant dry weight. Although none of the authors speculated on the possible reason of the shoot development repression by IAA, it could be due to enhanced apical dominance effect or simply the concentration of IAA used being inhibitory to differentiation of new leaf needles. According to the apical dominance phenomenon, exogenously applied IAA could have increased the dormancy of lateral buds hence reducing leaf needle development.

In this study, IAA was shown to play a role in mycorrhiza establishment and maintenance, specifically linking the Hartig' net formation to the fungus' response to IAA by hyperbranching of hypae. The biosynthesis of IAA could be shown to be induced in the fungus by tryptophan as well as IAAld. The conversion of IAAld to IAA is dependent on oxidation, which is proposed to be perfomed by the newly described gene product of *ald1* of *T. vaccinum*. The gene has been shown to be inducible by ethanol as well as aldehydes, which corresponds to finding of consensus sequences for stress response elements in the promoter region. Taken together, a function of *ald1* in plant interaction is proposed. Two other *T. vaccinum ald* genes clustering with *ald1* in a phylogenetic tree open the possibility that during evolution, *ald1* became specifically necessary for IAA metabolism while the other genes may be involved in other stress response pathways. The use of *A. tumefaciens*-mediated fungal transformation in functional gene analysis demonstrated here will allow monitoring of expression of mycorrhizal-specific genes *in vivo*, which will help to enhance our knowledge on ectomycorrhizal symbiosis on a molecular level.

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EIGENSTÄNDIGKEITSERKLÄRUNG (DECLARATION)

Ich erkläre, dass ich die Dissertation "**Molecular characterization of a fungal aldehyde dehydrogenase in the *Tricholoma vaccinum*-spruce ectomycorrhiza**" selbständig und nur mit der darin angegebenen Hilfe verfasst habe. Die Dissertation wurde in keiner anderen Fakultät oder Universität eingereicht.

I hereby declare that this dissertation "**Molecular characterization of a fungal aldehyde dehydrogenase in the *Tricholoma vaccinum*-spruce ectomycorrhiza"** is an original work. I guarantee that the content of this dissertation has not, in part or whole, been accepted for the award of any Degree in any university or institution.

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