Trehalose in the bicoloured deceiver (*Laccaria bicolor*)

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“Many of life's failures are experienced by people who did not realize how close they were to success when they gave up.”

Thomas Alva Edison (1847 - 1931)

Gutachter:

1. Prof. Dr. Uwe Nehls
2. Prof. Dr. Barbara Reinhold-Hurek

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I Zusammenfassung

Hefen sind Organismen, die auf Glukose-reiche Umgebungen spezialisiert sind und erst beginnen, die Kohlehydratspeicher Trehalose und dem gegenüber Glykogen anzulegen, wenn es zum Glukosemangel kommt (Walker 1998; Santangelo 2006). Die Gattung *Laccaria* bildet diese beiden Speicherzucker, wie viele Basidiomyceten und als wichtigen Bestandteil der arttypischen Ektomykorrhiza-Symbiose, im Gegensatz dazu bereits während einer konstanten Glukoseversorgung.

Für Asco- und Basidiomyzeten sind die Abstimmung von Zuckeraufnahme, Speicherung und die Verteilung auf die verschiedenen zellulären Stoffwechselwege essentiell. Diese Koordination wird höchstwahrscheinlich über verschiedene Sensorwege vermittelt, um extrazelluläre und intrazelluläre Zuckerquellen effizient zu nutzen, aber auch um den gesamten Stoffwechsel an die jeweiligen Umweltbedingungen anzupassen. Ein zentraler Mechanismus scheint das Zusammenspiel von Hexokinase und Trehalose-6-phosphat Synthase (TPS1) zu sein, die gemeinsam vermutlich die Glykolyse, den Pentosephosphatweg, die Kohlenhydratspeicherung (in Form von Trehalose und Glykogen) und die Zellwandbiosynthese steuern (Abb. 1).

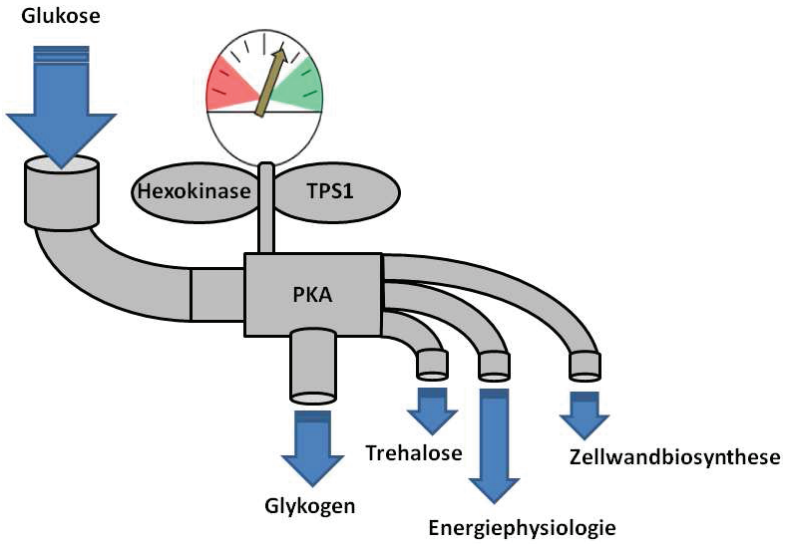


Abb. 1. Steuerung der Glukoseverteilung in *Laccaria bicolor*
Das Schema fasst die bisherigen Erkenntnisse aus Asco- und Basidiomyzeten zusammen. Die Trehalose-6-phosphat Synthase (TPS1) reguliert zusammen mit einer Hexokinase den Glukosefluss in die verschiedensten Stoffwechselwege. Diese Steuerung scheint maßgeblich über die Beeinflussung der Proteinkinase A (PKA) zu funktionieren.

Ein anscheinend zentrales Element dieser Regulation des Stoffwechsels ist die Proteinkinase A (PKA), die durch Phosphorylierung zum Einen die indirekt die Transkription und zum Anderen direkt die Aktivität von Enzymen kontrolliert.

Das Ziel dieser Doktorarbeit war die Manipulation des Kohlenhydratstoffwechsels des zur Ektomykorrhiza befähigten Basidiomyzeten *Laccaria bicolor*. Zu diesem Zweck wurde eine RNA Interferenz Strategie gewählt (RNAi), um gezielt die Transkription von Schlüsselenzymen des Trehalosemetabolismus (Trehalose-6-phosphate Synthase, Neutrale Trehalase, Trehalosephosphorylase) und des Glykogenmetabolismus (Glycogensynthase) zu hemmen. Zuerst wurde ein auf *Agrobacterium tumefaciens* basierendes Transformationssystem etabliert, das die Erzeugung von transgenen monokaryonten *Laccaria* Stämmen ermöglicht. Die Transformation von kreuzungskompatiblen Monokaryen wurde gewählt (S238N-H70 und S238N-H82), um im späteren gezielt unterschiedliche Kombinationen dikaryontischer Pilze zu erzeugen. Solche Hybridpilze ermöglichen somit die Kombination von verschiedenen RNAi-Konstrukten für die gezielte Repression von zwei unterschiedlichen Genen. Normalerweise ist die Kreuzung von Pilzen ein sehr arbeitsintensiver und zeitaufwändiger Vorgang, der allerdings im Rahmen dieser Doktorarbeit wesentlich effizienter gestaltet werden konnte. Das entwickelte Selektionssystem für erzeugte Dikaryen basiert auf optimierten Wachstumsbedingungen und einer identifizierten Ammonium-Sensitivität, das unabhängig von dominanten Selektionsmarkern funktioniert.

Da im Rahmen einer Doktorarbeit eine Fragestellung fokussiert bearbeitet werden soll, wurde das weitere Hauptaugenmerk auf die hergestellten TPS1-RNAi Transformanten gelegt. Es wurden die Auswirkungen des TPS1-RNAi auf a) das Wachstumsverhalten der Pilze und b) auf die Transkription von verschiedenen Genen, die eine Schlüsselstellung im Speicherkohlenhydratmetabolismus haben, untersucht.

Die mit dem TPS1-RNAi Konstrukt transformierten Pilze der beider genetischen Hintergründe zeigten vergleichbar starke Reduktionen in ihrer TPS1 Genexpression. Allerdings war das Bild, in Bezug auf Wachstumsparameter (Hyphendichte, maximale Wachstumsgeschwindigkeit) sowie die Hitzestress-Toleranz, uneinheitlich. Dieser Unterschied zwischen den beiden Pilzstämmen resultiert vermutlich aus einer Pheromon-vermittelten Abstimmung von Kohlenhydratversorgung mit der übrigen Pilzphysiologie.

Abschließend kann festgehalten werden, dass ein hefeähnliches Regulationssystem in *Laccaria* präsent ist, jedoch scheinen sich Basidiomyzeten in ihrem Reaktionsverhalten in einigen zentralen Punkten von den Ascomyzeten zu unterscheiden. Versorgt man

eine Kohlenhydrat-verarmte Hefekultur mit frischer Glukose, so werden die Speicherkohlenhydrate sofort abgebaut. In Basidiomyzeten hingegen werden diese Speicher (Trehalose und Glykogen) nach Zuckerzugabe aufgebaut, wobei für Glykogen häufig eine kurze zeitliche Verzögerung zu beobachten ist. Die Grundlage dieser Modulation der Kohlenhydratspeicher ist bei Ascomyzeten sehr gut untersucht. Hier bildet die cAMP-aktivierte PKA das Schlüsselelement zur Abstimmung der physiologischen Antwort auf die diversen Umweltstimuli. Diese zentrale Regulation scheint in Basidiomyzeten ebenfalls vorhanden zu sein, allerdings mit kleinen Unterschieden in der Feineinstellung der Physiologie an die spezifischen Umweltanforderungen.

II Summary

Yeast is well adapted to glucose rich environments and starts to accumulate trehalose and glycogen only during initial glucose depletion (Walker 1998; Santangelo 2006). In contrast, essential for ectomycorrhizal lifestyle, a typical feature of the genus *Laccaria*, is storage carbohydrate accumulation under conditions where cellular carbohydrates are not limiting (carbon sink formation in symbiosis). In both cases, channelling of external and internal carbohydrate resources into metabolism is central for cellular physiology. Different glucose sensing mechanisms are therefore supposed to be active in fungi to adapt cellular physiology to a changing environment. The complex interplay of hexokinase and trehalose-6-phosphate (TPS1) protein together with generation and consumption of hexose phosphates is expected to drive glucose fluxes into glycolysis, pentose phosphate shunt, storage carbohydrate metabolism (trehalose and glycogen), and cell wall biosynthesis (Fig. 2).

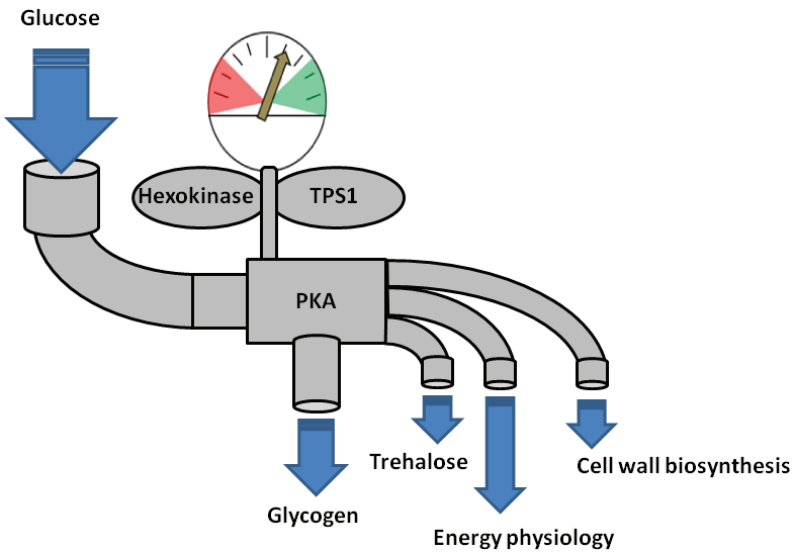


Fig. 2. Scheme of potential sugar sensing mechanism in *Laccaria bicolor*
As indicate by studies of basidiomycotic fungi, trehalose-6-phosphate synthase 1 (TPS1) together with hexokinase regulates the glucose flux into different metabolic pathways via the activity of proteinkinase A (PKA).

A central set screw of carbohydrate metabolism is proteinkinase A (phosphorylation) activity that transforms signals at two levels: transcription and posttranslational modification.

The aim of this thesis was to manipulate carbohydrate storage metabolism in the ectomycorrhizal basidiomycete *Laccaria bicolor*.

A RNA interference approach (RNAi) was established with the aim to decrease transcript levels of key enzymes of trehalose (trehalose-6-phosphate synthase 1, neutral trehalase, trehalose phosphorylase) and glycogen (glycogen synthase) metabolism. The first step was the adaptation of *Agrobacterium tumefaciens*-based transformation to monokaryotic *Laccaria* strains. Monokaryons of two genetics backgrounds were chosen because of their ability to fuse to dikaryotic mycelia (strains S238N-H70 and S238N-H82). The advantage of using monokaryons was the possibility to combine two different RNAi targets in single cells by having only one proven selection marker available for *Laccaria* transformation. Selection of dikaryotic mycelia after fusion of compatible monokaryons is, however, work and time consuming. By combining growth behaviour and ammonium phosphate toxicity, a fast and highly efficient selection procedure was established.

Due to time restriction, the main focus of the further work was on the characterisation of TPS1-RNAi transformants. Two attempts were followed to determine the impact of the TPS1-RNAi expression on fungal physiology: a) mycelial growth behaviour and b) characterisation of transcript levels of genes coding for proteins involved in storage carbohydrate metabolism.

While suppression of TPS1 expression by RNAi was comparable in the two monokaryotic *Laccaria* strains, reduced growth speed, hyphal density, and heat stress resistance were only observed for transformants with S238N-H70 background. Thus strain-specific differences in pheromone-based regulation of storage carbohydrate metabolism can be assumed for *Laccaria bicolor*.

While all regulatory set screws known to control carbohydrate metabolism in yeast can be supposed to be present in *Laccaria*, basidiomycetes seem to differ from ascomycetes in their response behaviour. While storage carbohydrates are degraded upon glucose addition to carbohydrate starved yeast cells, trehalose and, sometimes with short retardation, glycogen accumulation is the typical response of *Basidiomycota*. cAMP-based activation of PKA activity has been frequently described for ascomycetes to be a central trigger to integrate several environmental cues into physiological answers. Apart from fine tuning of certain responses, cAMP-based repression of PKA activity could explain the different behaviour of basidiomycetes.

1 Introduction

1.1 Bicoloured deceiver (*Laccaria bicolor*) as model organism

Scientific interest in fungal physiology started many decades ago. Knowledge on fungal metabolic processes was mainly derived from research performed in baker's yeast (*Saccharomyces cerevisiae*), while nowadays, selection of fungal model organisms has strongly increased because many research interests like fungal diseases, symbiotic interactions, as well as certain saprophytic capacities cannot be addressed using *S. cerevisiae* (Walker 1998).

When thinking about distinct physiological capabilities of certain fungi, their phylogenetic relationship often has to be taken into account (Fig. 3 and 4).

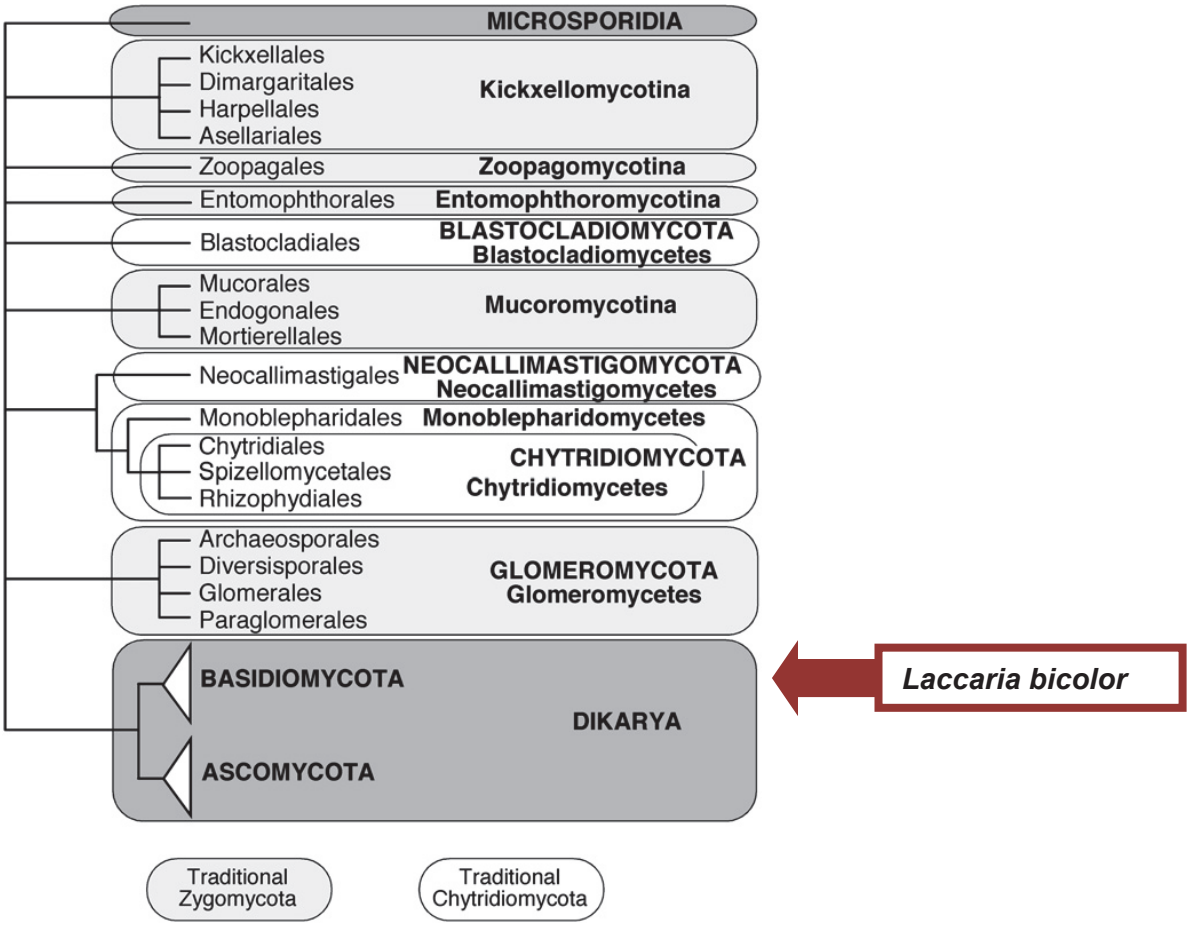


Fig. 3. Phylogenetic classification of the fungal kingdom (adapted from Hibbett, Binder et al. 2007)
The non-proportional phylogenetic tree includes monocellular, filamentous, and fruiting body forming fungi. The tree indicates the relationships between the different fungal orders. In the subkingdom of Dikarya, *L. bicolor* is grouped into the phylum *Basidiomycota*.

Laccaria belongs to the Dikarya that can be separated from all other fungal classes because of their predominant lifecycle in which septated hyphae do contain two nuclei of compatible mating type (Sitte, Weiler et al. 2002). There are of course certain exceptions as some ascomycetous yeasts or the yeast-like basidiomycete *Cryptococcus* are monocellular in major phases of their life, which start filamentous growth only by specific environmental stimuli (Edgington, Blacketer et al. 1999; Gancedo 2001; Lin and Heitman 2005; Lin 2009).

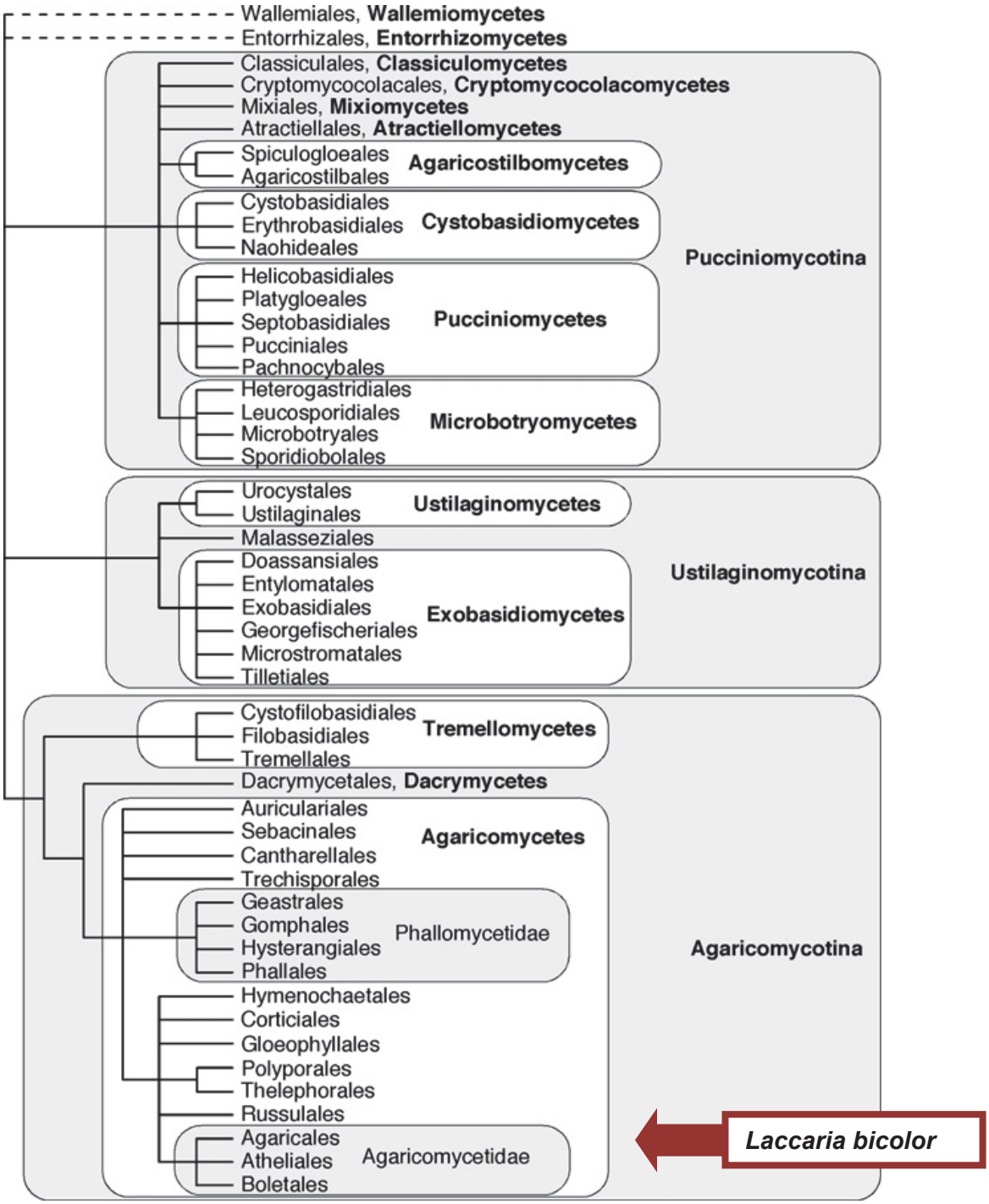


Fig. 4. Basidiomycotic fungal families and their relationships (Hibbett, Binder et al. 2007, adapted)
Demonstrated is a phylogenetic rearrangement of known basidiomycetes, based on recently achieved molecular phylogenetic data. The red arrow indicates the phylogenetic position of *Laccaria bicolor*.

Within the *Basidiomycota*, which are defined through a specific structure of their spore-releasing meiosporocysts, the basidium (see life cycle in 1.1.1), *L. bicolor* belongs to the subphylum *Agaricomycotina* and here to the subclass *Agaricomycetidae* (Matheny, Curtis et al. 2006). In this subclass, the important order of *Agaricales* is located comprising families with strong importance in food industry like the white mushroom *Agaricus bisporus* or the oyster mushroom *Pleurotus ostreatus*. Within the *Agaricales*, the genus *Laccaria* is positioned in the family *Hydnangiaceae* (Matheny, Curtis et al. 2006) as shown in Fig. 4.
Laccaria bicolor (Maire; P. D. Orton) was one of the first basidiomycotic fungi where the entire genome has been sequenced (Martin, Aerts et al. 2008). Its genome is composed of about 65 megabases with approximately 20,000 predicted protein coding genes. As typical for higher basidiomycetes, a very large proportion of the DNA consists of sequence repeats, including transposons. Compared to the genomes of basal basidiomycotic fungi, like *Ustilago maydis* (Kamper, Kahmann et al. 2006) or *Cryptococcus* (Loftus, Fung et al. 2005), the genome of *Laccaria* is characterised by the amplification of certain gene families involved in communication (effector-type small secreted proteins; SSPs) and regulation (Martin, Aerts et al. 2008; Martin and Selosse 2008).

1.1.1 Lifecycle of *Basidiomycota*

Primary goal of every living being is survival and reproduction. In Fig. 5 a scheme of the lifecycle of a typical fruiting body forming basidiomycete is shown. It starts with the germination of a generative basidiospore, forming a haploid mycelium where septated hyphae do contain only one nucleus (termed monokaryons). As soon as another monokaryotic mycelium of a compatible mating type is available, cells fuse and a mycelium where each cell does contain two haploid nuclei (termed dikaryons) is formed (Sitte, Weiler et al. 2002; Lull, Wichers et al. 2005). Under natural conditions, typically dikaryotic hyphae of basidiomycotic fungi are found and certain developmental capabilities are rather inefficient (e.g. formation of ectomycorrhiza, see below) or even impossible (fruiting body formation) for monokaryotic mycelia (Erke 1976; Kropp, McAfee et al. 1987; Kropp and Fortin 1988; Wong, Piché et al. 1989; Di Battista, Selosse et al. 1996; Schmidt 1996; Smith and Read 2008). The fruiting body of the

most common Agaricomycetes is composed of a stem and a gill containing cap in which heterogenesis, basidiospore formation, and spore release occur.

Environmental conditions are expected to trigger fruiting body formation (Hall, Yun et al. 2003). Especially CO_2 concentration, humidity, salinity (Flegg, Spencer et al. 1985; Wessels 1993; Ohga 1999; Kues and Liu 2000), and the pH of the applied substrates are such factors. Furthermore, initiation of mushroom formation can be suppressed by light (Richartz and Maclellan 1987; Wessels 1993; Kues 2000; Kues and Liu 2000). Fruiting body formation can only take place if sufficient resources (carbohydrates, nutrients) have been stored within the fungal mycelium. In *Laccaria,* as an ectomycorrhizal fungus (see below), fruiting body formation is only possible when a fungal colony is connected to a plant partner (Kropp, McAfee et al. 1987; Kropp and Fortin 1988; Gay, Normand et al. 1994; Di Battista, Selosse et al. 1996; Niculita-Hirzel, Labbe et al. 2008).

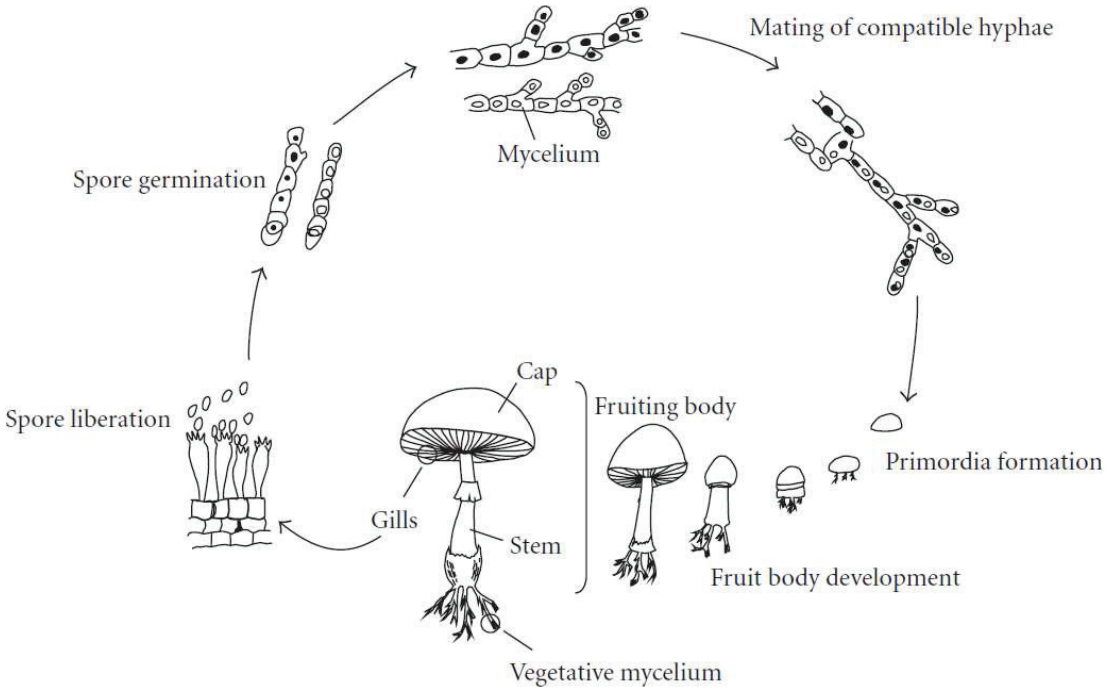


Fig. 5. Typical lifecycle of higher *Basidiomycota* (adapted from Lull, Wichers et al. 2005)
After basidiospore germination a haploid mycelium will be formed that will fuse (mate) with cells of a compatible partner rather soon. The dikaryotic mycelium, where each cell harbours nuclei of both parents, is the most prominent form of these fungi and can grow in this form forever. Under certain conditions generative organs are formed, frequently visible as mushrooms. Mushrooms can be structured into stem and cap where basidia are formed. In these basidia karyogamy as well as meiosis will occur. Thereafter, the novel haploid nuclei will migrate into mostly four stem-like structures, where the formation of basidiospores takes place.

1.1.1.1 The tetrapolar mating system of *Laccaria*

Mating of higher fungi is based on a cytoplasmic fusion of two haploid cells of compatible mating types. However, fungi often do have a more complex crossing scheme than just two sexes. In more than half of all known *Agaricomycotina* the mating process is controlled by many different alleles of two unlinked regions in the fungal genomes of both parental nuclei. These loci are termed as the *A* and *B* loci. Thousands of different combinations might potentially result by combinations of these alleles to manage the mating in such fungi of which only some are compatible (Koltin, Stamberg et al. 1972; Kues and Casselton 1992; Kues and Liu 2000; Raudaskoski and Kothe 2010). Terminating the system as tetrapolar was done by the means of four different allelic combinations (two for each partner) that are necessary for functional mating. In case of successful mating of theoretically incompatible combinations, the generated dikaryons are often (nearly 75 %) unable to sporulate and stay as sterile dikaryotic mycelia. Such semi compatible reactions occur, if *A*-types are different and *B*-types identical or *vice versa* (Kothe 2001). In most *Basidiomycota*, this behaviour allows fertile crossing among over 90 % of non-related individuals of a species, whereas inbreeding among sibling strains is reduced to 25 % (Raper 1966).

The *A* mating type locus contains genes for two types of homeodomain transcription factors (HD1 and HD2), while the *B* mating type locus encodes lipopeptide pheromones and G-protein-coupled pheromone receptors (STE3-like). A successful mating of *Agaricales* seems to require the formation of heterodimeric transcription factors (HD1 plus HD2) which act as a master switch of correct mating (Banham, Asante-Owusu et al. 1995; Niculita-Hirzel, Labbe et al. 2008). In dikaryotic mycelia *A*-type heterodimeric transcription factors regulate paring of nuclei, synchronise nuclear division, initiation of clamp cell formation, and septation. Such clamp cell formation is a complex process required for proper partitioning of both nuclei during cell division (see Fig. 6). As shown in various experiments, pheromones and their receptors (*B* locus) are essential for partner recognition prior to fusion of monokaryotic hyphae, as well as both reciprocal nuclear exchange and migration directly after hyphal fusion (Reginald Buller 1924; Whitehouse 1949; Hesler and Petersen 1971; Kothe 2001; Heitman 2007; Niculita-Hirzel, Labbe et al. 2008; Raudaskoski and Kothe 2010). In fully developed dikaryotic hyphae, *B* gene products are needed for peg formation, fusion of the hook cell to the subapical cell, and nuclear migration which completes the patch clamp function (Badalyan, Polak et al. 2004; Raudaskoski and Kothe 2010). The interplay of *A* and *B* mating type genes is furthermore required for fruiting initiation and karyogamy

(Moore 1998; Boulianne, Liu et al. 2000; Kues 2000; Kues and Liu 2000; Badalyan, Polak et al. 2004; Srivilai and Loutchanwoot 2009), see Fig. 6.

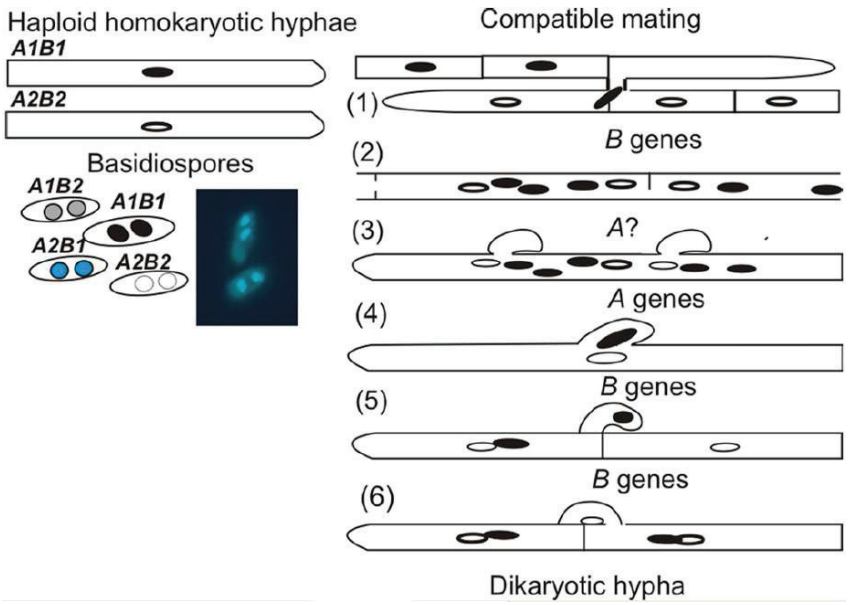


Fig. 6. Mating of compatible homokaryotic hyphae of basidiomycotic fungi (adapted from Raudaskoski and Kothe 2010)
On the left side, the basidiospores are illustrated with all four potential mating type genes (*A1, A2, B1,* and *B2*) arranged in pairs. These spores will form the haploid monokaryotic mycelium illustrated in the left top of the figure. In (1) the mating of two compatible hyphae is shown, which compatibility is defined through different *A*- and *B*-type genes in both monokaryons. Multinuclear hyphae are the first fusion products directly after the mating (2). *B*-type genes are responsible for correct formation of clamp connections and also lead to the correct arrangement (3) of both nuclei forming the dikaryotic mycelium (4). *B* genes inhibit *A* function in cases of incorrectly sorted nuclei. In (5) and (6) the propagation of dikaryotic hyphae is indicated via the establishment of functional clamp connection for nucleus separation, followed by a septation of the clamp.

In *Laccaria bicolor* 45 *A* and 24 *B* mating type alleles are known defining the tetrapolar mating system, a situation quite similar to the findings in *C. cinerea* (Raudaskoski and Kothe 2010). Both highly conserved HD transcription factors of the *A* locus are divergently regulated in the sequenced monokaryotic *Laccaria* strain S238N-H82 (Raffle, Anderson et al. 1995; Niculita-Hirzel, Labbe et al. 2008). The *B* locus of this strain encodes 13 functional STE3-like pheromone receptors and three pheromones. Transcripts of all receptor-like genes were detectable but transcript levels of three of them, which are in close proximity to pheromone genes, were much higher in vegetative mycelium, ectomycorrhiza, and fruiting bodies (Niculita-Hirzel, Labbe et al. 2008).

As consequence of pheromone-receptor interaction, cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) pathways, heterotrimeric G proteins, and

mitogen-activated protein kinase (MAPK) cascades are involved in mating (hyphal fusions, septal dissolution, clamp formation, and clamp fusion) and fruiting body development (Raudaskoski and Kothe 2010). Furthermore, cell division cycle protein 42 (CDC42) was elucidated to function in linking extracellular growth signals to actin cytoskeleton during the clamp fusion. CDC42 acts in parallel to nuclear positioning via microtubule modulation at the fusion point (Raudaskoski, Rupes˘ et al. 1991; Raudaskoski 1998; Kothe 1999; Raudaskoski and Kothe 2010).

1.1.2 *Laccaria bicolor* - an ectomycorrhiza forming model fungus

Approximately 30 % of soil-located forestal microbial biomass is formed by fungi (*Zygo*-, *Asco*-, or *Basidiomycota*) that are, in part, associated with fine roots of (mainly) trees, forming together a new structure, the ectomycorrhiza (ECM) (Molina, Massicotte et al. 1992; Wallander, Nilsson et al. 2001; Högberg and Högberg 2002; Wallander 2006). This association is essential for adequate nourishment of trees in frequently nutrient limited forest ecosystems and is thus of large ecological and economical importance. Plant nutrition can be improved because of the capability of ectomycorrhizal fungi to mobilise nutrients that are either of organic (soil organic matter) or inorganic (minerals) origin which cannot be directly used by forest trees themselves (Chalot, Blaudez et al. 2006; Nehls 2008; Smith and Read 2008). Further benefits of ectomycorrhizal mutualism are an increased resistance against drought and pathogens (Marx 1970; Pankow, Boller et al. 1991; Schelkle and Peterson 1997; Garbaye 2000). However, these advantages are not for free. The plant partner delivers up to 30 % of its total assimilates to fungal partners (Söderström and Read 1987; Söderström 1992; Jones and Darrah 1996; Farrar and Jones 2000; Högberg and Högberg 2002; Hobbie 2006). In Fig. 7 a scheme of an ectomycorrhizal relationship is shown (Nehls 2008). Soil growing hyphae take up minerals and water and deliver them to the plant partner through specialised transport hyphae (rhizomorphs), while carbohydrates from ectomycorrhiza are transported towards the growing mycelial front (Nehls 2008; Smith and Read 2008). The ECM (right part of Fig. 7; (Nehls 2008)) is composed of two fungal networks, an root-shielding hyphal mantle and an apoplastic hyphal network (between the root cortical cells), termed Hartig net that is responsible for nutrient and metabolite exchange.

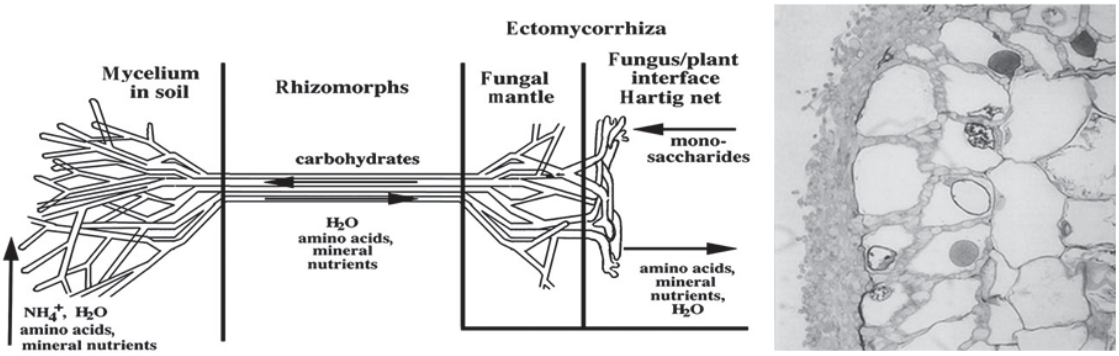


Fig. 7. Scheme of a colony of a typical ectomycorrhizal fungus (adapted from Nehls 2008) Soil living hyphae (left) are responsible for uptake of water and nutrients for fungal nutrition, which are frequently distributed within the colony via fungal rhizomorphs. Ectomycorrhiza (right part of the figure), another part of the colony, are composed by a fungal mantle and a hyphal network growing within the apoplast of the root cortex, the so-called Hartig net, where nutrient and metabolite exchange between plant and fungus occur.

The reason for choosing *Laccaria bicolor* as a model for ectomycorrhizal research, more than 30 years ago, was the easy way of its cultivation and ectomycorrhiza formation under laboratory conditions. During this time, a clear picture of its symbiotic capabilities, mating behaviour, and overall physiology (Graham and Linderman 1980; Molina 1982; Fries 1983; Kropp, McAfee et al. 1987; Kropp and Fortin 1988; Wong, Piché et al. 1989; Gardes, Fortin et al. 1990; Bastide, Kropp et al. 1995; de la Bastide, Kropp et al. 1995; Martin, Aerts et al. 2008; Martin and Selosse 2008; Niculita-Hirzel, Labbe et al. 2008) has been drawn. The presence of a nuclear sequence together with the capability of transformation make this fungus to one of the best currently available *Laccaria* derived models for a symbiotic organism. This is further promoted by the recent discovery of key factors necessary for successful establishment of mycorrhizal symbiosis (Plett, Kemppainen et al. 2011; Plett and Martin 2012).

1.1.3 Carbohydrate acquisition in ectomycorrhizal fungi

Basidiomycotic mycobionts do not seem to have the ability to degrade complex plant cell wall material to efficiently cover their carbohydrate demand (Trojanowski, Haider et al. 1984; Haselwandter, Bobleter et al. 1990). This was recently confirmed by nuclear genome analyses of *Laccaria bicolor* and other ECM fungi, where no genes coding for enzymes involved in cellulose or hemicellulose degradation were obtained. To be competitive within soil ECM fungi therefore strongly depend on the constant delivery of easy degradable carbohydrates by the plant partner (Lewis and Harley 1965; Cairney, Ashford et al. 1989; Leake, Donnelly et al. 2001; Wu, Nara et al. 2002; Nehls and

Bodendiek 2012). Such plant released carbohydrate sources are proteins, organic acids, amino acids, or simple sugars (Harley and Smith 1983; Smith and Read 2008).
All so far analysed ectomycorrhizal fungi are not able to exploit sucrose as no fungal invertase activity or sucrose uptake were ever observed (Salzer and Hager 1991; Schaeffer, Wallenda et al. 1995; Hatakeyama and Ohmasa 2004; Daza, Manjon et al. 2006), a feature that is again supported by genome data of *L. bicolor* (Martin, Aerts et al. 2008). ECM fungi, however, have a well developed capability to utilise monosaccharides (Harley and Smith 1983). In agreement, certain genes coding for functional proton-coupled monosaccharide importers were strongly expressed upon ectomycorrhizal symbiosis (Nehls, Wiese et al. 1998; Wiese, Kleber et al. 2000; Nehls 2004; Polidori, Ceccaroli et al. 2007; Lopez, Dietz et al. 2008; Nehls 2008). Moreover, the identification of a novel class of plant transporters capable in monosaccharide efflux, termed SWEETS (Chen, Hou et al. 2010; Baker, Leach et al. 2012; Braun 2012; Chen, Qu et al. 2012), further supports the assumption that glucose is the main fungal carbohydrate source in ECM symbiosis (Nehls and Bodendiek 2012; Nintemann 2012).

1.2 Carbohydrates in fungi

1.2.1 Sugar phosphorylation

Conversion of hexoses into their phosphates ensures a continuous concentration gradient of hexoses between intra- and extracellular space (Bisson and Fraenkel 1983; Rose, Albig et al. 1991). Therefore, hexose kinases are thought to be key players for regulation of sugar uptake and carbohydrate channelling into metabolism and storage. In baker's yeast (*Saccharomyces cerevisiae*) three glucose-phosphorylating (at C6 position) enzymes were described, hexokinase 1 (HXK1) and 2 (HXK2), which both phosphorylate fructose and glucose, and glucokinase (GLK1) which is specific for glucose (Beullens and Thevelein 1990; Rose, Albig et al. 1991; Clifton, Walsh et al. 1993). HXK2 is the main glucose-phosphorylating enzyme when cells are growing under glucose-rich conditions, while HXK1 is most active when glucose is scarce. Repression of high-affinity hexose transporter genes is observed when yeast cells are exposed to abundant glucose concentrations (Ozcan and Johnston 1995). In addition, triple *HXK1, HXK2, GLK1* mutants lack high-affinity glucose uptake capability (Bisson and Fraenkel 1984). Therefore, a feedback regulation of hexose kinases on transcription of sugar transporters and enzyme activity was supposed (Bisson and Fraenkel 1983; Bisson, Coons et al. 1993; Ko, Liang et al. 1993). HXK2 activity

seemed to be necessary for intracellular glucose sensing (Bisson and Kunathigan 2003).

Phosphorylated sugars can be either directed towards primary metabolism/energy production (glycolysis, pentose phosphate shunt) or towards carbohydrate storage. With regard to basidiomycotic fungi, carbon storage is of special importance as carbohydrates are frequently distributed over long distances within the fungal colony. Concerning fungal function in ECM, the formation of storage carbohydrates is expected to be essential for strong carbon sink strength formation in symbiosis. Efficient transfer of large amounts of plant carbohydrates into the fungal mycelium is supposed to take place only when imported glucose is quickly converted (e.g. into storage compounds, see below). Nevertheless, apart from gene expression relative little is known about sugar phosphorylation in ECM fungi.

1.2.2 Glycogen and trehalose in fungi

Glycogen and trehalose have overlapping (carbohydrate storage) but also distinct (stress response) functions in fungi and some trehalose functions can be taken over by sugar alcohols, for instance mannitol (Nehls 2008). The focus of this work, however, will mainly be on glycogen and trehalose, because sugar alcohols seem to have more minor functions in *Laccaria*.

Trehalose and glycogen share their dependency on UDP-glucose as precursor for biosynthesis. As UDP-glucose is (indirectly) formed from glucose-6-phosphate, primary and storage carbon metabolism are linked by this sugar phosphate (Fig. 8) (François, Walther et al. 2012).

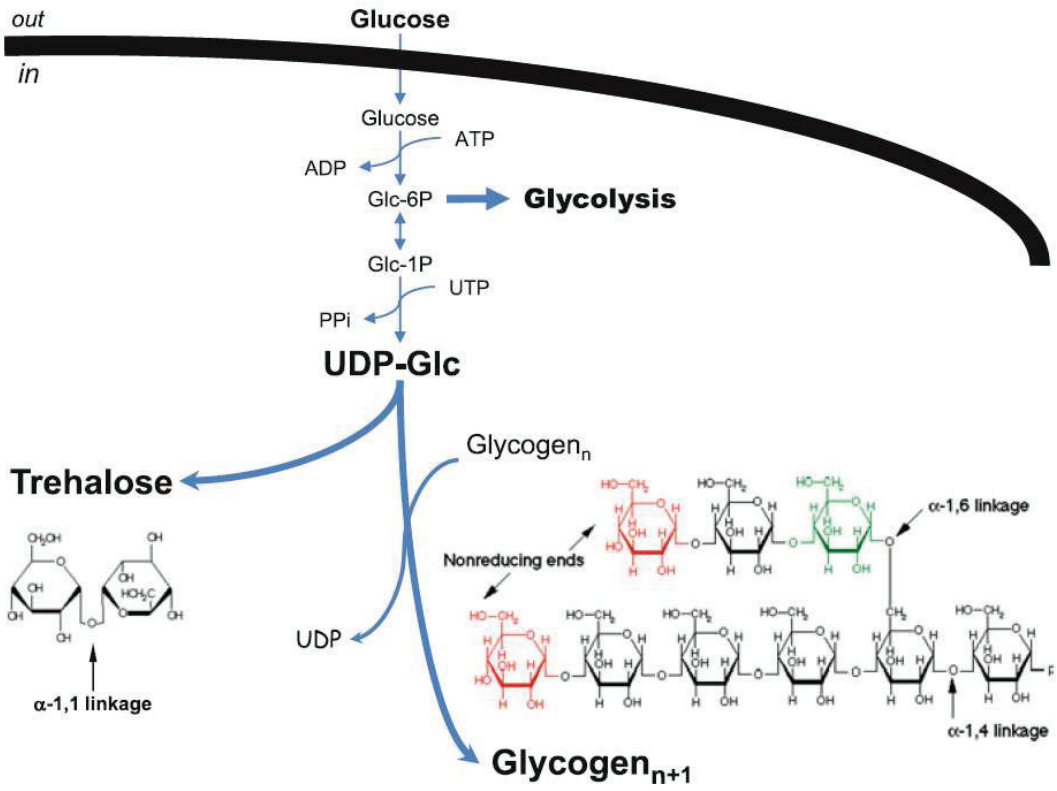


Fig. 8. Glucose destinations in *S. cerevisiae* (adapted from François, Walther et al. 2012) The uptake of glucose by yeast cells is connected with its phosphorylation to glucose-6-phoshate that links primary (glycolysis) and carbohydrate storage (trehalose, glycogen) metabolism.

1.2.2.1 Glycogen metabolism

Glycogen metabolism is well conserved over the whole fungal kingdom. Glycogen consists of several thousand glucose monomers (Roach, Cao et al. 1991; Melendez, Melendez-Hevia et al. 1999) and is usually located in the fungal cytosol. Its synthesis is initiated by a protein termed glycogenin, which generates a short chain of α-(1,4)-glucosyl residues (glycogen primer) attached to a tyrosine by auto-glucosylation activity using UDP-glucose as substrate (Alonso, Lomako et al. 1995; Baque, Guinovart et al. 1997; Lomako, Lomako et al. 2004; François, Walther et al. 2012). After detachment of the primer from glycogenin, the elongation of glycogen is accomplished by the glycogen synthase, which couples glucose residues to the non-reducing end of glycogen (then α-(1,4)-linked), using UDP-glucose as donor. Within the genome of *S. cerevisiae*, two genes (*GSY1* and *GSY2*), sharing 80 % sequence identity, are found of which *GSY1* seems to be the predominant active glycogen synthase (Farkas, Hardy et al. 1990; Farkas, Hardy et al. 1991). A specific branching enzyme (encoded by *GLC3*) *Saccharomyces* completes glycogen synthesis by forming of α-(1,6)-bridges. The

protein covalently connects the non-reducing end of a chain of seven α-(1,4)-glucosyl units from one linear glycogen molecule to the C6 position of another (branched) glycogen molecule (Thon, Vigneron-Lesens et al. 1992; Alonso, Lomako et al. 1995).
High molecular glycogen is mainly degraded by two cytosolic enzymes, glycogen phosphorylase (encoded by *GPH1* in yeast) and glycogen debranching enzyme (encoded by *GDB1*). Glycogen phosphorylase's activity is regulated by cyclic AMP-mediated phosphorylation (PKA) and its allosteric inhibitor glucose-6-phosphate. This enzyme was determined to be a key player of glycogen degradation in yeast, generating glucose-1-phosphate (conservation of the glycosidic bound energy) as product. Deletion of *GPH1* resulted in a higher cellular glycogen content. However, trehalose metabolism was unchanged in this mutants and the GPH1 function was determined as not essential for yeast survival (Hwang, Tugendreich et al. 1989; Lin, Hwang et al. 1995; Lin, Rath et al. 1996). The glycogen debranching enzyme acts as α-(1,4)-glucanotransferase and α-(1,6)-glucosidase, finally generating glucose as product. Deletion of *GDB1* resulted in an inhibited glycogen breakdown (Teste, Enjalbert et al. 2000). Furthermore, a glucose generating amylo-glucosidase (encoded by *SGA1* in yeast) is localised within vacuoles, which hydrolyses 1,4- and 1,6-connected glucosyl residues (Colonna and Magee 1978; Clancy, Smith et al. 1982). This enzyme is mainly expressed during sporulation or under carbon starvation (Clancy, Smith et al. 1982; Teste, Duquenne et al. 2009). The enzyme is supposed to avoid uncontrollable glycogen degradation in the cytosol by using a separated glycogen pool surrounded by the tonoplast (Wang, Wilson et al. 2001; François, Walther et al. 2012).

1.2.2.2 Trehalose metabolism

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide in which an α,α-(1,1)-glycosidic linkage connects two glucose units. It is widely distributed over all kingdoms of living organisms (bacteria, plants, fungi, and animals) (Avonce, Mendoza-Vargas et al. 2006). Five pathways were described so far to generate the disaccharide (Avonce, Mendoza-Vargas et al. 2006) but only two are described here in detail because of their occurrence in fungi.

1.2.2.2.1 Trehalose biosynthesis by the TPS-complex

The pathway mainly responsible for trehalose formation in fungi is mediated by two enzymes and was intensively discovered using *Saccharomyces cerevisiae* (Avonce, Mendoza-Vargas et al. 2006). As first step, trehalose-6-phosphate is synthesised from

UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (TPS1 or TPS) and then dephosphorylated by trehalose-6-phosphate phosphatase (TPS2 or TPP) (Leloir and Cabib 1953; Cabib and Leloir 1958; Panek 1959; Panek 1962; Thevelein and Hohmann 1995). This pathway is known to be highly conserved among the fungal kingdom. In yeast, deletion of *TPS1* completely inhibits the synthesis of trehalose (Van Aelst, Hohmann et al. 1993; Thevelein and Hohmann 1995), while deletion of *TPS2* results in an accumulation of trehalose-6-phosphate (De Virgilio, Burckert et al. 1993).

Trehalose-6-phosphate synthase is regulated by phosphorylation, leading to deactivation of enzyme activity. The presence of micromolar amounts of ATP could partially overcome this phosphorylation-dependent inactivation of TPS1 (Panek, de Araujo et al. 1987). K_m values for glucose-6-phosphate and UDP-glucose were determined as approximately 3.5 mM and 0.5 mM. Non-competitive inhibition of the reaction was achieved by Pi, UDP, and UTP. Trehalose-6-phosphate phosphatase of yeast revealed K_m values of close to 0.2 mM at pH 6.0 and 0.5 mM at pH 7.0 as well as a Mg^{2+} dependency (Vandercammen, Francois et al. 1989). It is commonly accepted that TPS1 and TPS2 together with two other proteins (TSL1 and TPS3) form a so-called TPS-complex to become fully functional (Fig. 9).

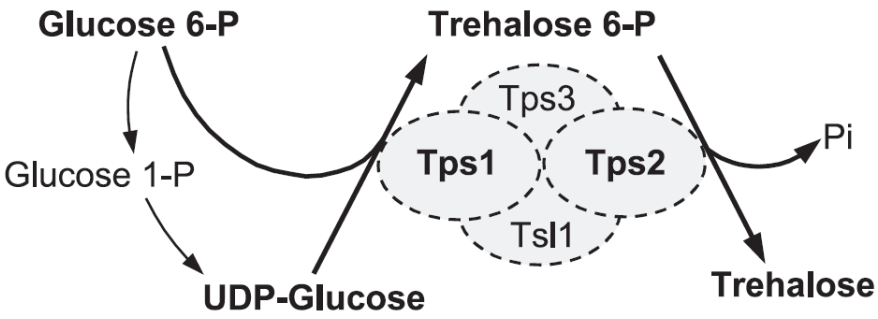


Fig. 9. TPS-complex of *Saccharomyces cerevisiae* (from Gancedo and Flores 2004)
The *Saccharomyces* TPS-complex consists of four different subunits (grey ellipses). In this complex TPS1 (Tps1; trehalose-6-phosphate synthase) and TPS2 (Tps2; trehalose-6-phosphate phosphatase) catalyse trehalose biosynthesis. Tps1 uses glucose-6-phosphate (Glucose 6-P) and UDP-Glucose to generate trehalose-6-phosphate (Trehalose 6-P) which will be afterwards dephosphorylated by the Tps2 to obtain trehalose. The two other subunits Tps3 (TPS3) and Tsl1 (TSL1) are regulatory and structural elements of the complex without enzyme activity.

TSL1 and TPS3 share 55 % identity at the protein level and interact with TPS1 and TPS2 but not directly with each other (Vuorio, Kalkkinen et al. 1993; Reinders, Burckert et al. 1997). *TPS3/TSL1* double mutants displayed a significantly reduced trehalose biosynthesis and isogenic deletions of *TSL1* or *TPS3* revealed an interchangeable

regulatory function of both proteins. However, TPS1 alone is able to synthesis trehalose-6-phosphate, but trehalose synthesis is less efficient under such circumstances (Reinders, Burckert et al. 1997; Bell, Sun et al. 1998).

1.2.2.2.2 Trehalose metabolism by trehalose phosphorylase

In basidiomycotic fungi a trehalose phosphorylase (TP) was described, which is able to catalyse the reversible phosphorolysis of trehalose in the presence of inorganic phosphate, resulting in the formation of glucose-1-phosphate and glucose (Kitamoto, Akashi et al. 1988; Saito, Yamazaki et al. 1998; Wannet, Op den Camp et al. 1998). Based on the fact that no TPS-complex or trehalase activities were detectable, this enzyme was discussed to be responsible for fungal trehalose metabolism in *Agaricus* fruiting bodies (Saito, Yamazaki et al. 1998; Wannet, Op den Camp et al. 1998). Both trehalose degradation and biosynthesis have an optimum between pH 6.0 and 7.0. Trehalose degradation by TP was inhibited by ATP and trehalose analogues, whereas biosynthesis was inhibited by phosphate. As K_m values were 61 mM for trehalose, 4.7 mM for phosphate, 24 mM for glucose, and 6.3 mM for glucose-1-phosphate, hyphal metabolite concentrations make TP's phosphorolytic activity most likely under normal conditions (Saito, Yamazaki et al. 1998; Wannet, Aben et al. 1999; Han, Kwon et al. 2003).

1.2.2.2.3 Degradation of trehalose by trehalases

Trehalose degradation occurs via hydrolysis resulting in two molecules glucose (Metzenberg 1962; Hill and Sussman 1964; Mandels, Vitols et al. 1965; Mandels and Vitols 1967; Souza and Panek 1968; Gibson, Gloster et al. 2007). Main reasons for trehalose degradation are glucose utilisation for growth, fruiting body formation (Mandels, Vitols et al. 1965; Mandels and Vitols 1967; Souza and Panek 1968; Kuenzi and Fiechter 1969; Londesborough and Varimo 1984; Thevelein, den Hollander et al. 1984; Wannet, Op den Camp et al. 1998), or recovery of natural cell physiology after stress-caused trehalose accumulation (Londesborough and Varimo 1984; Thevelein 1984; Hottiger, Schmutz et al. 1987; Ocon, Hampp et al. 2007). Two trehalose-hydrolysing activities, divided by their predominant pH values, were described in fungi, named as acid and neutral trehalases. Acid trehalases have their highest activity at pH 4.5-5.0 and are located either in the vacuole or the apoplast, whereas neutral trehalases are most active at pH 6.8-7.0 and are located in the cytosol (Keller, Schellenberg et al. 1982; Londesborough and Varimo 1984; Thevelein 1984; Harris and Cotter 1988; Mittenbuhler and Holzer 1988; App and Holzer 1989; Alizadeh and

Klionsky 1996). In most fungal organisms several isoforms of trehalases are described (Parrou, Jules et al. 2005; Wolska-Mitaszko, Jaroszuk-Scisel et al. 2007; Deveau, Kohler et al. 2008). Extracellular trehalose can be hydrolysed by apoplastic acid trehalase, ATH1 in *Saccharomyces* (Jules, Beltran et al. 2008), while neutral cytosolic trehalases, NTH1 and NTH2 in *Saccharomyces* (Kopp, Muller et al. 1993; Wolfe and Lohan 1994; Jules, Beltran et al. 2008), are responsible for degradation of intracellular trehalose either after accumulation or import by a trehalose transporter, AGT1 in *Saccharomyces* (Jules, Guillou et al. 2004). However, the inability of yeasts to growth on trehalose after deletion of *ATH1* demonstrates its particular role also in degradation of extracellular trehalose (Nwaka, Mechler et al. 1996). However, NTH1 was demonstrated to be most active in mobilisation of endogenous trehalose as gene deletion resulted in a 75 % loss of measurable cytoplasmic trehalase activity. Purified neutral trehalase was identified as homodimer with K_m values for trehalose between 5-35 mM, depending on the phosphorylation state (activation via phosphorylation and *vice versa*) (Londesborough and Varimo 1984; Dellamora-Ortiz, Ortiz et al. 1986; App and Holzer 1989; Francois and Parrou 2001). Compared to neutral trehalase, yeast acid trehalase has a higher affinity for trehalose (K_m 1.5-4.5 mM) (Londesborough and Varimo 1984; Mittenbuhler and Holzer 1988; Francois and Parrou 2001).

1.2.3 Carbohydrates as fungal storage compounds

Most fungi analysed so far produce two main storage carbohydrates: glycogen and trehalose. Trehalose and glycogen accumulation was observed in resting cells (Thevelein 1984) and spores (Lillie and Pringle 1980) of yeast or *Aspergillus nidulans* (Fillinger, Chaveroche et al. 2001), where both carbohydrates together can reach up to 25 % of the dry weight. Also in spores of arbuscular mycorrhizal fungi trehalose accumulates (Smith, Muscatine et al. 1969; Bécard, Doner et al. 1991).

Trehalose and glycogen are fast degraded after spore germination (Kane and Roth 1974; Colonna and Magee 1978; Thevelein 1984) and, at least in yeast, during restart of cell growth in nutrient-rich medium (Thevelein 1984; Francois, Neves et al. 1991). Glycogen levels starts to decrease also, when nutrients are depleted. Such a pattern is typical for carbohydrate storage to ensure continuous glucose supply (Lillie and Pringle 1980; Francois, Villanueva et al. 1988; Francois, Neves et al. 1991; Francois and Parrou 2001; Jules, Beltran et al. 2008).

Basidiomycotic fruiting bodies contain large amounts of trehalose and glycogen. Trehalose and mannitol accumulation seems to be the basis for fast expansion of fruiting bodies (increase in cell volume as consequence of elevated osmolarity) and is

important for spore formation (Wallenda 1996; Wannet, Aben et al. 1999; Heleno, Barros et al. 2011). In vegetative mycelia of basidiomycetes, trehalose and glycogen levels increase when hyphae are grown in glucose rich medium. In contrast to yeast, storage carbohydrate accumulation starts immediately when hyphae are transferred into carbohydrate containing medium (Francois and Parrou 2001).

1.2.3.1 Trehalose as carbon store in ectomycorrhizal symbiosis

Trehalose accumulation was observed in *Pisolithus tinctorius* when grown on glucose as sole carbon source. However, similar trehalose accumulation was also observed after successful mycorrhiza development of the fungus, indicating trehalose to be important in glucose sink formation in symbiosis (Ineichen and Wiemken 1992; Martin, Boiffin et al. 1998). The potential role of trehalose in carbon sink formation is further promoted by studies in *Amanita muscaria* (Lopez, Manner et al. 2007). Compared with the extra-radical mycelium, the expression of genes coding for TPS1, TPS2 (TPP), and TP were strongly (18-fold, 3.5-fold, and 4-fold, respectively) increased in hyphae at the plant-fungus interface (Hartig net) of functional ectomycorrhizae. Accordingly, TPS1 enzyme activity was 7.4-fold and trehalose content 2.7-fold increased in Hartig net hyphae (Lopez, Manner et al. 2007).

Apart from local carbohydrate storage, trehalose is also supposed of having a function in long distance carbohydrate transport within the fungal colony (Söderström, Finlay et al. 1988; Nehls 2008). Trehalose export towards other parts of the fungal colony would also further increase the carbohydrate sink strength at the plant fungus interface, because it ensures efficient glucose conversion without product (trehalose) feedback inhibition. Furthermore, based on the known strong impact of trehalose and trehalose-6-phosphate on plant physiology, fungal trehalose release was also discussed as a way to modulate root cell behaviour in mycorrhizal symbiosis (Wiemken 2007).

1.2.4 Function of trehalose in fungi in response to abiotic stress

Trehalose accumulation has been frequently observed in yeast as a response to heat, cold, desiccation, osmotic and oxidative stress, and during exposure to toxicants (Hottiger, De Virgilio et al. 1994; Voit 2003; Kandror, Bretschneider et al. 2004; Zancan and Sola-Penna 2005; Conlin and Nelson 2007; Li, Ye et al. 2009). Membrane (Crowe, Crowe et al. 1984; Jain and Roy 2009) as well as a protein stabilisation was described as putative mechanism (Müller, Boller et al. 1995). In acquired thermo-tolerance the proportion of surviving yeast cells is correlated to intracellular trehalose levels (Attfield 1987; Hottiger, Boller et al. 1987; Hottiger, Schmutz et al. 1987; Hottiger, Boller et al.

1989; Wiemken 1990). Yeast cells accumulate trehalose up to 17-fold (up to nearly 16 % of cell dry weight) at temperatures below 10 °C (Kandror, Bretschneider et al. 2004) or above 36 °C and are then able to survive freezing as well as temperatures of up to 50 °C (Hottiger, Boller et al. 1987; Hottiger, Boller et al. 1989; Wiemken 1990; Ribeiro, Silva et al. 1994). Accordingly, transcript levels of *TPS1* and *TPS2* together with certain heat shock proteins strongly increased within 30 minutes after temperature shock. *TPS1* (in *Emericella nidulans* (Fillinger, Chaveroche et al. 2001) and yeast (De Virgilio, Burckert et al. 1993)) and *TPS2* (in yeast (De Virgilio, Burckert et al. 1993)) mutants were highly sensitive to elevated temperatures (above 37 °C). During stress recovery NTH1 activity increased, resulting in rapidly reduced intracellular trehalose levels (Li, Ye et al. 2009).

The importance of trehalose as stress protectant was also described in ectomycorrhizal fungi. *Hebeloma* uses trehalose and partially sugar alcohols to survive cold periods. During pre-conditioning of mycelia to cold temperature, trehalose accumulates up to 2.5 % of mycelial dry weight (Tibbett, Sanders et al. 2002). Trehalose content was also indentified to correlate with spruce mycorrhizal vitality (Niederer, Pankow et al. 1989) under drought stress and during winter term freezing (Niederer, Pankow et al. 1992). In addition, in the arbuscular mycorrhizal fungus *Glomus intraradices* heat and chemical stress were further identified to induce accumulation of trehalose (Ocon, Hampp et al. 2007). A transient up-regulation of the *TPS2* transcription as well as enzyme activity was discovered as stress response. Moreover, with regard to heat shock recovery and thermo tolerance, activities of neutral trehalase in *G. intraradices* were comparable to those of yeast (Ocon, Hampp et al. 2007).

Based on an initial plant defence reaction, the establishment of mycorrhiza itself was often discussed to be a kind of stress and fungal trehalose accumulation during early infection is supposed to be a consequence to increase fungal survival rate. Indeed, in endomycorrhizal symbiosis (*Glomus mosseae* and soybean) trehalose accumulated after colonisation of plant roots (Hoekstra, Crowe et al. 1992; Evelin, Kapoor et al. 2009) and mycorrhizal trehalose content correlated positively with the fungal infection rate.

1.2.5 Regulation of fungal carbohydrate metabolism

Transfer of carbohydrate starved cells to a glucose rich medium (Cannon, Gibbs et al. 1986; Cameron, Levin et al. 1988; Portela, Van Dijck et al. 2003) result in a synchronized trehalose and glycogen degradation in yeast, while major trehalose and glycogen accumulation was observed in late logarithmic and early stationary growth

phase and upon temperature stress. cAMP levels were demonstrated to be a major trigger for regulation of storage carbohydrate metabolism. Under normal conditions cAMP levels are very low in yeast cells and increase only transiently when environmental changes occur (Mbonyi, van Aelst et al. 1990; Portela, Van Dijck et al. 2003). Mutations in genes coding for proteins involved in cAMP-dependent protein kinase signalling strongly affected glycogen and trehalose accumulation (Uno, Matsumoto et al. 1983; Matsumoto, Uno et al. 1985; Cannon, Gibbs et al. 1986; Cannon, Pringle et al. 1994). Disruption of *TPS1* in yeast did not only prevented cAMP raise after glucose addition (Gonzalez, Stucka et al. 1992) but caused even overall decreased cAMP levels (Noubhani, Bunoust et al. 2009), resulting in a mutant defective in growth on glucose. Exorbitant accumulation of sugar phosphates were observed after glucose addition in this yeast mutant (Van Aelst, Hohmann et al. 1991; Hohmann, Neves et al. 1993; Van Aelst, Hohmann et al. 1993; Neves, Hohmann et al. 1995; Díaz-Ruiz, Avéret et al. 2008), resulting in a depletion of ATP, Pi, and all glycolytic intermediates downstream of glyceraldehyde-3-phosphate dehydrogenase (Hohmann, Bell et al. 1996). Introduction of the *OTSA* gene, the *Escherichia coli TPS1* homologue, into the *TPS1* deletion strain of *S. cerevisiae*, resulted in normal TPS activity as well as wild typical trehalose-6-phosphate levels. However, yeast growth on glucose was only partially restored and temporary hyperaccumulation of sugar phosphates was still observed immediately after glucose addition. Therefore a function of TPS1 in control of glucose flux into glycolysis was supposed (Bonini, Van Vaeck et al. 2000). In contrast to *OTSA*, expression of the *Aspergillus nidulans TPS1* homologue (*TPSA*) fully complemented the yeast *TPS1* mutant phenotype (Fillinger, Chaveroche et al. 2001).

The phenotype of the TPS1 mutant could be, however, partly restored by deletion of hexokinase 2, indicating a distinct role not only of TPS1 but also of HXK2 in glucose sensing. The interaction of TPS1 and hexokinase 2 function is mediated by trehalose-6-phosphate (T6P). In agreement with the supposed function of T6P, deletion of TPS2 resulted in increased cAMP levels and could be restored to wild type levels after deletion of hexokinase 2 in a TPS2 mutant background (Noubhani, Bunoust et al. 2009). Interestingly, after 50-fold overexpression of the hexokinase 2 no increased HXK2 activity was detectable (Ernandes, De Meirsman et al. 1998), indicating a direct TPS1/HXK2 interaction necessary for restriction of glucose influx by trehalose-6-phosphate (Noubhani, Bunoust et al. 2000).

Apart from its enzymatic and sensor function, HXK2 is directly involved in regulation of gene expression. In the presence of glucose, HXK2 is transferred into the nucleus

where it mediates a) glucose repression (e.g. *HXK1, GLK1,* and *SUC2*) and b) glucose induction (e.g. *HXK2* and some glucose importers) of several genes (de la Cera, Herrero et al. 2002; Moreno and Herrero 2002).

Similar to the situation in yeast, cAMP levels increased temporarily after transfer of carbohydrate starved mycelia into glucose rich medium in the ectomycorrhizal basidiomycete *Amanita muscaria* (Hoffmann, Wallenda et al. 1997). However, contrasting the situation in yeast, rise in cAMP levels resulted in a strong increase in cellular trehalose content in *Amanita* (Hoffmann, Wallenda et al. 1997). Furthermore, such fast and massive trehalose formation as response to fresh glucose was often demonstrated for ectomycorrhizal basidiomycetes (Wallenda 1996; Samborski 2012).

1.3 RNA interference as a tool to manipulate gene expression in *Laccaria*

Induced target mRNA degradation via triggered RNA interference (RNAi) is a common tool in animals (Fire, Xu et al. 1998; Sharp 1999; Qadota, Inoue et al. 2007; Kleinhammer, Deussing et al. 2011), plants (Smith, Singh et al. 2000; Burgos-Rivera and Dawe 2012), and fungi (de Jong, Deelstra et al. 2006; Costa, Mills et al. 2008; Costa, Thomas et al. 2009) to manipulate gene expression. The common strategy for RNAi-induced target gene silencing (also known as PTGS, posttranscriptionally gene silencing) is the formation of double-stranded RNA (dsRNA), complementary to the target mRNA (Schwab, Ossowski et al. 2006) within the organism of interest. This dsRNA will be recognised and hydrolysed into smaller fragments, about 21 nucleotide in length termed as small interfering RNA (siRNA), by an enzyme complex called DICER RNase. The Argonaute protein of the RNA-induced silencing complex (RISC) binds such siRNAs, which are then used to target the respective complementary mRNA and to degrade it (Elbashir, Lendeckel et al. 2001; Hannon and Rossi 2004; Filipowicz 2005; Schwab, Ossowski et al. 2006).

To enable the formation of double-stranded RNAs necessary for RNAi, the respective organisms are frequently transformed with a construct containing an inverted repeat of the coding region of the target gene that is transcribed under the control of a strong promotor. In eukaryotes such inverted repeats have to be separated by a spliceable intron sequence for efficient RNAi induction. When transcribed, the complementary sequences of the inverted repeat will hybridise, forming so-called hairpin RNA molecules with an extended double stranded region. The splicing process of the intron region is supposed to increase the recognition and hydrolysis of the remaining double-

stranded RNA molecule by the DICER-RNase (Smith, Singh et al. 2000; Hannon and Rossi 2004; Kemppainen, Duplessis et al. 2009). A simplified scheme of the construction of such gene-specific inverted repeats for RNAi is shown in Fig. 10.

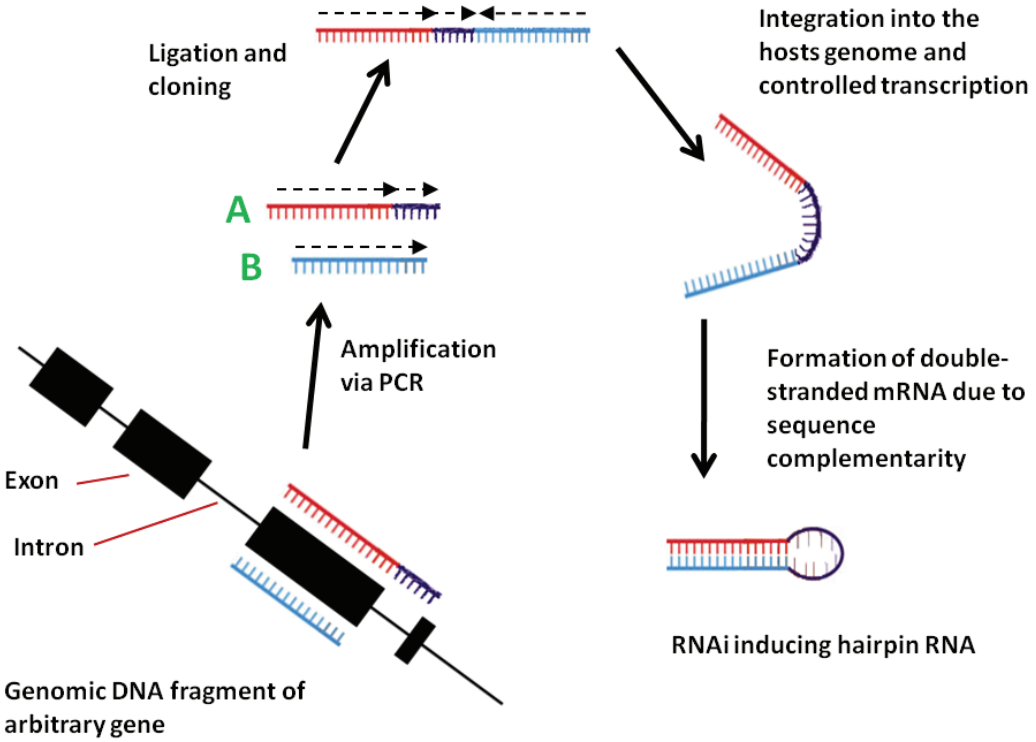


Fig. 10. Construction scheme of gene-specific inverted repeats for RNAi
First step to generate RNAi inducing constructs is the PCR amplification of two genomic DNA fragments that will form the inverted repeat separated by an intron. Two DNA fragments of the same exon will be amplified, one containing only the exon (B; light blue) and the other containing the exon and an intron at its 3'-end (A; exon highlighted in red, intron in lilac). Both fragments are ligated in a way that the intron-containing fragment will be in sense and the intron free fragment in antisense orientation (as indicated by the small broken arrows above the fragments). When transcribed, such a construct will form a so-called hairpin RNA. Here, complementary RNA regions are forming an extended double strand, separated by an intron-containing loop structure that will be spliced off later.

All proteins required for RNAi-based gene silencing were determined in the *Laccaria* genome and first successful demonstrations of the power of this technique in *Laccaria* were performed (Kemppainen, Duplessis et al. 2009).

1.4 Aim of the thesis

The aim of this thesis was the investigation of distinct functions of the storage carbohydrates glycogen and trehalose in *Laccaria bicolor,* with special emphasis to trehalose metabolism. Trehalose is of special interest for ectomycorrhizal fungi because of its supposed dual function (sink formation and long distance transport) in

symbiosis. Therefore, trehalose biosynthesis (TPS1) and degradation (NT) were aimed to be targeted by RNA interference approaches. Because of its unclear function in trehalose metabolism, trehalose phosphorylase (TP) was chosen as additional minor target. With regard to glycogen metabolism, glycogen synthase (GS) for biosynthesis was selected.

As currently only a single selection marker is known for *Laccaria* transformation, compatible monokaryotic strains of the fungus were chosen for transformation. Transformants harbouring different combination of gene suppression constructs should be combined by dikaryon formation to allow the suppression of several aspects of trehalose and glycogen metabolism.

For characterisation of RNAi-based gene suppression and interpretation, investigation of fungal growth and expression of genes, coding for proteins involved in storage carbohydrate metabolism, were planned to be determined.

2 Materials and Methods

2.1 Bioinformatics

2.1.1 Programs and databases

Tab. 1. Sequence analysis and manipulation programs

Software	Function	Source
Gene Runner version 3.05	Primer design	Hastings Software, Inc., Hastings, NY, USA
Chromas Lite version 2.01	Analysis of sequencing data	http://www.technelysium.com.au/, Technelysium Pty. Ltd., South Brisbane, Australia
DNASTAR Lasergene® Seqbuilder version 7.1.0	Vector design, sequence analysis	www.dnastar.com, DNASTAR, Inc., Madison, WI, USA
Clustal X version 2.1	Sequence alignments, calculation of phylogenetic trees	www.clustal.org, (Thompson, Gibson et al. 1997; Larkin, Blackshields et al. 2007)
BioEdit version 7.0.9.0	Sequence manipulation	http://www.mbio.ncsu.edu/BioEdit/bioedit.html, Tom Hall, Ibis Biosciences, Carlsbad, CA, USA
Treeview 1.6.6	Analysis of phylogenetic trees	http://taxonomy.zoology.gla.ac.uk/rod/treeview.html, (Page 2002)
Archaeopteryx 0.972+ beta 9M	Analysis of phylogenetic trees	https://sites.google.com/site/cmzmasek/home/software/archaeopteryx (Han and Zmasek 2009)

2.1.2 Online sources

Tab. 2. Selected databases for identification of trehalose-dependent proteins
Tabled are selected organisms (with used abbreviations in brackets) with corresponding online sources applied for protein alignments and dendrogram creation. The last access in context of this PhD research was 10.01.2013.

Organism	Database
Agaricus bisporus var. bisporus H97 (Abi)	http://genome.jgi.doe.gov/Agabi_varbisH97_2/Agabi_varbisH97_2.home.html
Arabidopsis thaliana Columbia 0 (Ath)	http://www.ncbi.nlm.nih.gov/guide/
Aspergillus niger (Ani)	http://genome.jgi-psf.org/Aspni5/Aspni5.home.html http://www.uniprot.org/uniprot/
Belgica antarctica (Ban)	http://www.ncbi.nlm.nih.gov/guide/
Coprinopsis cinerea 7#130 (Cci)	http://www.broadinstitute.org/annotation/genome/coprinus_cinereus/MultiHome.html
Emericella nidulans (Eni)	http://www.ncbi.nlm.nih.gov/guide/ http://www.uniprot.org/uniprot/
Escherichia coli (Eco)	http://www.ncbi.nlm.nih.gov/guide/
Laccaria bicolor S238N-H82 (Lbi)	http://genome.jgi-psf.org/Lacbi2/Lacbi2.home.html
Neurospora crassa OR74A (Ncr)	http://www.broadinstitute.org/annotation/genome/ne

	urospora/MultiHome.html
Pleurotus ostreatus (Pos)	http://genome.jgi-psf.org/PleosPC15_2/PleosPC15_2.home.html
Pleurotus pulmonaris (Ppu)	http://www.ncbi.nlm.nih.gov/guide/
Pleurotus sajor-caju (Psa)	http://www.ncbi.nlm.nih.gov/guide/
Saccharomyces cerevisiae S288C (Sce)	http://www.yeastgenome.org/
Schizophyllum commune (Sco)	http://www.ncbi.nlm.nih.gov/guide/
Ustilago maydis BroadModels1 (Uma)	http://mips.helmholtz-muenchen.de/genre/proj/ustilago/

2.1.3 Identification of genes involved in trehalose metabolism

Until the primary release of the genome sequence (version 1.0) of *Laccaria bicolor* (Maire) P. D. Orton strain S238N-H82 and the first annotation of genes involved in fungal carbon metabolism by Deveau et al. (2008), intense resequencing and gap filling occurred. Therefore, a second approach to screen the annotated *Laccaria* genome for genes involved in trehalose and glycogen metabolism was performed. Rescreening of the current *Laccaria* genome was conducted using the web-based analysis tools BROWSE (search by predicted chromosome scaffolds or protein IDs), KEGG (proteins sorted by metabolic pathways), and KOG (proteins sorted by functional classification) of the *Laccaria* database (Tab. 2). Furthermore, as novel information about proteins involved in trehalose metabolism of other fungi (such as *Saccharomyces cerevisiae*, *Ustilago maydis*, *Agaricus bisporus, Neurospora crassa*, *Aspergillus niger,* and *Pleurotus ostreatus*), and the thale cress (*Arabidopsis thaliana*) were published (for all selected organisms with corresponding genome databases see Tab. 2), intensive BLAST (Altschul, Madden et al. 1997) analysis of the current *Laccaria* genome version (2.0) was performed for selected aspects. To get some clues about regulation of trehalose and glycogen metabolism in *Laccaria*, a protein BLAST search for homologues of known *Saccharomyces cerevisiae* transcription factors (Martinez-Pastor, Marchler et al. 1996; Winderickx, de Winde et al. 1996; Gorner, Durchschlag et al. 1998; Thevelein and de Winde 1999; Sunnarborg, Miller et al. 2001) was also performed.

The local computer programs Clustel X 2.1, Bioedit 7.0.9.0, Treeview 1.6.6, Archaeopteryx 0.972+ beta 9M, and DNASTAR Lasergene® Seqbuilder version 7.1.0 (see Tab. 1) were used for protein alignments and dendrogram drawings.

2.2 Statistical analysis

Statistical analysis was performed by Student's paired t-test for two tailed distributions and unequal variances of data using Excel 2007 (Microsoft Corporation, Redmond, WA, USA). P-values were considered as: significant (*, p≤ 0.05), highly significant (**, p≤ 0.01), and extremely significant (***, p≤ 0.001).

2.3 Organisms and plasmids

2.3.1 Organisms

Laccaria bicolor (Maire; P. D. Orton) strains: S238N*;* S238N-H82*;* S238N-H70*;* and S238N-H70xH82 were provided by Prof. Dr. F. Le Tacon and Prof. Dr. F. Martin (INRA – Centre de Nancy, Champenoux, France).

Agrobacterium tumefaciens strains: AGL1 (Lazo, Stein et al. 1991), C58/PMP90 (Koncz and Schell 1986), and LBA 4404 (Hoekema, Hirsch et al. 1983) were used.

Escherichia coli strains: SURE (*endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC e14- Δ(mcrCB-hsdSMR-mrr)171 F'[proAB*$^{+}$ *lacI*^{q} *lacZΔM15 Tn10];* (Agilent Technologies, Waldbronn, Germany) and TOP 10 F' (*F'[lacI*^{q} *Tn10(tet*^{R}*)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str*^{R}*) endA1λ*$^{-}$ (Invitrogen, Groningen, The Netherlands) were utilised.

2.3.2 Plasmids

2.3.2.1 *E. coli* cloning vectors

The cloning vectors pJet1.2/blunt (NCBI accession number EF694056) and pBluescript II SK (+) (NCBI accession number X52328) were obtained from Fermentas GmbH (St. Leon-Rot, Germany); vector maps and functional elements are demonstrated in the appendix Fig. 65 and 66.

The vector pHP45Ω (Prentki and Krisch 1984) was kindly provided by Dr. Andrea Krause (Department of Plant-Microbe Interactions, University of Bremen, Bremen, Germany; Fig. 11).

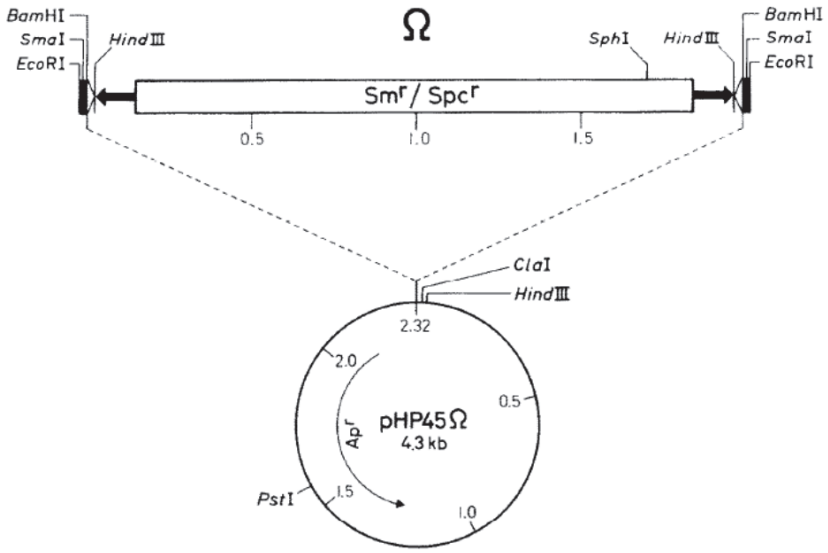


Fig. 11. Vector map of pHP45Ω (adapted from Prentki and Kirsch 1984)
Bacterial spectinomycin as well as streptomycin resistance cassette (Ω), with flanking restriction recognition sites enabling the release of cassettes, is highlighted.

2.3.2.2 The fungal transformation vector pBGgHg

The binary vector pBGgHg, pictured in Fig. 12 (Chen, Stone et al. 2000), was kindly provided by Dr. Roland Marmeisse (CNRS d'Ecologie Microbienne, Université Claude Bernard Lyon, Lyon, France).

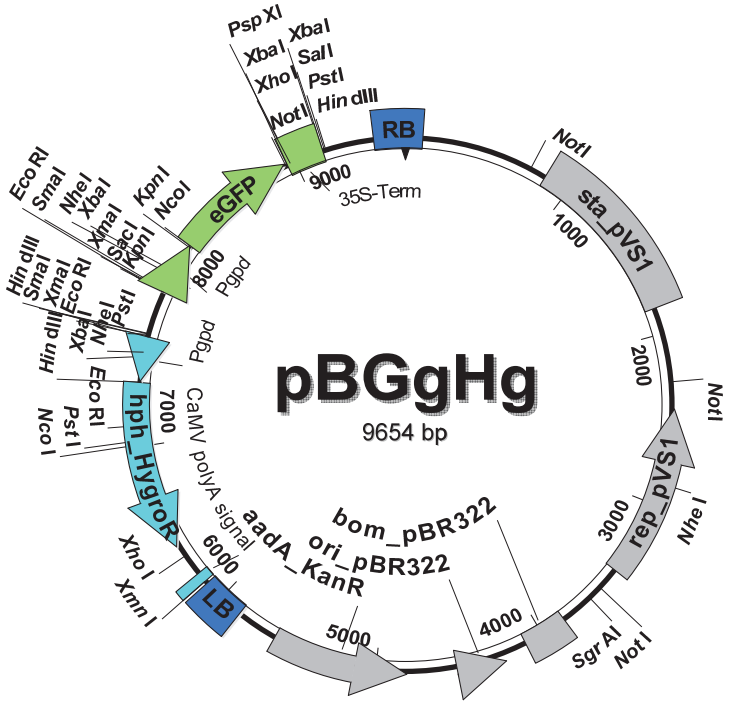


Fig. 12. Vector map of pBGgHg
Functional elements and restriction enzyme digestion sites of binary vector pBGgHg are shown; origin of replicon for *Agrobacterium* (rep_pVS1) derived from *Pseudomonas aeruginosa* pVS1 replicon, stability region derived from *Pseudomonas aeruginosa* pVS1 replicon (sta_pVS1), origin of replication for *Escherichia* from pBR322 (ori_pBR322), bom (basis of mobilisation) site from pBR322 required for transfer through conjugation (bom_pBR322), aminoglycoside 3'-phosphotransferase for kanamycin resistant (aadA_KanR), polyadenylation signal derived from CaMV (CaMV polyA signal), terminator of transcription (35S-term), enhanced green fluorescent protein (eGFP), glyceraldehyde-3-phosphate dehydrogenase promoter (Pgpd), hygromycin B phosphotransferase for hygromycin resistance in fungi (hph_HygroR), right t-DNA border sequence (RB), left t-DNA border sequence (LB).

2.4 Culture of organisms

All cultivation work was performed under sterile conditions. Different incubators, partly equipped with a shaker for liquid culture (see appendix Tab. 14), were used. Media and solutions were either autoclaved (at 121 °C for 20 min and 2 bar) or sterile filtered, if heat sensitive, using 0.2 µm pore size filters (CARL ROTH GmbH & Co. KG, Karlsruhe, Germany).

2.4.1 Bacteria

For *E. coli* culture (at 37 °C) LB (lysogeny broth) medium (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl), supplemented with antibiotics (ampicillin (100 mg/L), kanamycin, (50 mg/L), streptomycin (100 mg/L), or spectinomycin (100 mg/L)) and 18 g/L agar (Kobe 1) if necessary was used.

Agrobacterium was cultivated at 28 °C on King`s B medium (20 g/L peptone, 10 % (w/v) glycerol, 9 mM K_2HPO_4, 6 mM $MgSO_4$ x $7H_2O$, pH 7.2 equilibrated with phosphoric acid) supplemented with ampicillin (100 mg/L), kanamycin (50 mg/L), rifampicin (100 mg/L), or spectinomycin (100 mg/L) either on petri dishes (solidified with 18 g/L agar; Kobe 1) or in liquid culture. For fungal transformation YEB medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, and 2 mM $MgCl_2$) was also used.

2.4.2 *Laccaria bicolor*

Laccaria bicolor mycelia were grown on modified Melin Norkrans medium (MMN) (Marx 1969; Kottke, Guttenberger et al. 1987) either on petri dishes (with 18 g/L agar, Kobe 1) or in liquid culture, usually supplemented with 25 mM glucose. However, concentrations of 0.25 mM, 2.5 mM, 25 mM, or 100 mM glucose were used for special purpose. Inoculation of agar plates, if necessary covered by a sterile cellophane membrane (Einmachfix; Max Bringmann KG, Wendelstein, Germany), was carried out by transferring an approximately 3 x 3 mm agar block excised from the edge of a growing fungal colony and placing it in the middle of the fresh plate. 15-20 mycelial agar blocks served for inoculation of a liquid culture (70 mL MMN medium in a 300 mL flask) that was further cultivated under continuous shaking (120 rpm). The growth time ranged between 7 days and up to several moths at 18 °C in darkness. For storage of hyphal material, mycelia were freeze-dried through quick-freezing in liquid N_2 and subsequent lyophilisation (at -20 °C in a continuous vacuum of 5 x 10^{-3} mbar).

2.5 Molecular biological methods

2.5.1 Isolation of nucleic acids

2.5.1.1 Isolation of total RNA

All solutions and materials required for RNA working were autoclaved (40 min at 121 °C at 2 bar) or baked (180 °C for 4 h) to prevent RNase contamination.

Total RNA was isolated from up to 10 mg of freeze-dried fungal mycelium using the NucleoSpin RNA® Plant kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturers' instructions. Freeze-dried hyphae were ground with 20 mg glass beads (Ø 0.1 mm CARL ROTH GmbH & Co. KG, Karlsruhe, Germany) in 300 µL extraction buffer in 1.5 mL microreaction tubes by a rotating locked pestle driven by a rotary stirrer (500 rpm). DNase treatment was optimised for fungal hyphae

by adding 120 % of the DNase quantity proposed by the manufacturer. To test for remaining DNA contamination, quantitative real-time PCR (see 2.5.3.3) was performed. RNA quantification and estimation of protein contamination were carried out photometrically (Nano Drop™ DN1000; PEQLAB Biotechnologie GmbH, Erlangen, Germany).

For quality assessment of isolated RNA, agarose gel electrophoresis was carried out (see 2.5.3.1) and RNA was visualised using ethidium bromide staining (2.5.3.3).

2.5.1.2 Isolation of genomic DNA

About 200 mg of fresh or 20 mg of lyophilised fungal mycelium were ground under liquid nitrogen in a mortar with pestle to a fine powder. After addition of 1 mL lyses buffer (50 mM CTAB, 100 mM Tris-HCl, 26 mM EDTA, 1 mM NaCl, pH 8.0) the powder was homogenised by pestle agitation. The solution was transferred into a 2.2 mL reaction tube, incubated for 45 min at 60 °C under gent shaking and centrifuged for 5 min at 18,000 x g. The supernatant was transferred into a new 2.2 mL reaction tube and 500 µL chloroform: isoamyl alcohol (24:1) were added followed by vortexing to remove proteins, lipids and hydrophobic secondary metabolites. After centrifugation for 1 min at 18,000 x g the upper aqueous phase was transferred into a fresh 2.2 mL reaction tube. Then, 500 µL of ice cold isopropanol were added and mixed with the DNA solution by slow inversion of the reaction tube. After incubation for 1 h at -20 °C the genomic DNA was precipitated by centrifugation (5 min at 18,000 x g). The pellet was washed twice by addition of 100 µL ice cold 70 % (v/v) ethanol and centrifugation for 1 min at 18,000 x g, followed by removal of the supernatant by pipetting. The pellet was air-dried at room temperature for about 15 min, then dissolved in 35 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and subsequently incubated at room temperature for 5 min, at 50 °C for 5 min, and at 65 °C for 20 min. RNA removal was conducted by addition of 1 µL RNase A solution (5 µg/mL) and incubation for 20 min at 37 °C followed by ethanol precipitation by addition of 1/10 volume of sodium acetate (3 M, pH 5.0 equilibrated with acetic acid) and two volumes of ethanol (absolute) followed by brief vortexing. After incubation for at least 20 min at -20 °C, 1 µL of glycogen solution (5 mg/mL) was added and the DNA was precipitated by centrifugation (5 min at 18,000 x g). The DNA containing sediment was washed with 70 % (v/v) ethanol, air-dried, and dissolved in 35 µL TE buffer. Genomic DNA was kept either for few days at 4 °C (short term storage) or at -20 °C (long term storage). DNA quality and quantity were estimated by gel electrophoresis (0.8 % (w/v) agarose gels) together with a size marker and ethidium bromide staining (see 2.5.3.2 and 2.5.3.3).

2.5.2 Amplification of DNA fragments by PCR

PCR reactions (total volume 20 µL) were carried out with 0.2 µL proof-reading Taq polymerase (Phusion® DNA polymerase), 4 µL 5 x reaction buffer, 10 pmol of each primer, 0.2 µL dNTP mix (10 mM), and 100 ng of genomic DNA or 20 ng of plasmid DNA in a thermocycler (PEQLAB Biotechnologie GmbH or Biometra GmbH). For amplification of genomic DNA, a two step protocol (see 2.5.4.6; two fragments per gene) was performed to maximise PCR efficiency, for conditions see Tab. 3. Elongation times were calculated according to the estimated fragment length. An amplification rate of 1 Kbp per minute was supposed but elongation times never exceed 3 min.

Tab. 3. Conditions for amplification of genomic DNA

Cycle	Function	Time [min]	Temperature [°C]	
1	Initial denaturation for activating the polymerase	00:30	98	
2	Denaturation	00:10	98	
3	Primer annealing	00:10	52-60	**35 repeats**
4	Polymerisation	Maximum 03:00	72	
5	Terminal elongation	05:00	72	

2.5.3 Gel-electrophoresis of nucleic acids

2.5.3.1 RNA

RNA quality was determined by agarose gel electrophoresis. Thus, RNA samples were mixed with 1/5 vol. RNA loading dye (38 mM MOPS, 30 % (v/v) formamide , 8.75 % (v/v) formaldehyde, 20 % (w/v) glycerol, 0.01 % (w/v) bromophenol blue) and incubated at 50 °C for 5 min. Subsequently, mixtures were chilled on ice for 2 min and loaded onto formaldehyde agarose gels (1.5 % (w/v) agarose, 27.5 mM MOPS, 6.6 % (v/v) formaldehyde). As running buffer a 27.5 mM MOPS solution, supplemented with 12 mM Na-acetate and 1.25 mM EDTA (pH 7.0 equilibrated with acetic acid), was prepared. DEPC-treated water (1 mL DEPC per 1 L H_2Odd; stirred overnight at 37 °C) was used for preparation of agarose gels and running buffer. After electrophoresis, agarose gels were washed 4 times for 20 min with DEPC water prior to ethidium bromide staining (see 2.5.3.3.). Alternatively, RNA samples were mixed with loading

dye (as described above) and separated in 1.5 % (w/v) agarose gels using 1 x TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0 equilibrated with acetic acid) running buffer prior to ethidium bromide staining. DEPC water was used for preparation of all solutions.

2.5.3.2 DNA

DNA fragments were analysed by electrophoresis in agarose gels; 0.8 % (w/v) agarose for DNA fragments between 16 and 4 Kbp, 1 % (w/v) agarose for DNA fragments between 6 and 0.5 Kbp, and 2 % (w/v) agarose for DNA fragments below 0.5 Kbp were applied. 1 x TAE was always used as running buffer. *Eco130I*-digested lambda-phage (λ) DNA (Fermentas GmbH) or GeneRuler^{TM} 100 bp DNA Ladder (Fermentas GmbH) were used as a size standard.

2.5.3.3 Visualisation of DNA/RNA

Nucleic acids, separated in agarose gels, were incubated in ethidium bromide solution (0.2 ‰ (v/v)) and visualised by UV light (312 nm, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) using the DOC-PRINT II gel documentation camera system (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

2.5.4 Cloning of DNA fragments

2.5.4.1 DNA digestion

Analytic restriction analysis was conducted with approximately 1 µg of plasmid DNA, 4-10 U of restriction enzyme (Fermentas GmbH, St. Leon-Rot, Germany, or New England Biolabs GmbH (NEB), Frankfurt, Germany) and 2 µL (10 x) enzyme-specific buffer in a total volume of 20 µL at the desired temperature for 2-3 h.
For preparative purpose about 2-5 µg of plasmid DNA were incubated with 10-15 U restriction endonuclease and 3 µL nuclease-specific buffer (10 x) in a total volume of 30 µL at the desired temperature for 4 h.

2.5.4.2 Isolation of DNA fragments from agarose gels

After gel-electrophoretical DNA separation and ethidium bromide-based visualisation, DNA fragments of expected size were excised from the agarose gel under UV light illumination (312 nm wavelength) using a fresh scalpel. DNA containing excised agarose gel blocks were applied to the "NucleoSpin® Extract II Kit" according to the manufacturers' instructions.

2.5.4.3 Ligation of DNA fragments into plasmids

In case of single enzyme-based integration, vector DNA was dephosphorylated using FastAP™ thermosensitive alkaline phosphatase (Fermentas GmbH) according to manufacturer's protocol.

DNA fragments with sticky ends were ligated at 15 °C over night with 1 µL T4 DNA ligase (Fermentas GmbH) and 2 µL (10 x) ligase buffer in a total reaction volume of 20 µL. Vector/insert ratios between 1:3 and 1:4 were used.

Fragments with blunt ends were introduced to fast ligation reaction (5 min at room temperature) with 1 µL T4 DNA ligase plus 10 µL of 2 x reaction buffer in a total volume of 20 µL according to manufacturers' instructions (CloneJET™ PCR Cloning Kit). Vector/insert ratios between 1:1 and 1:3 were introduced for ligation.

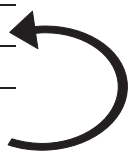
2.5.4.4 Plasmid preparation from *Escherichia coli*

A single plasmid carrying *E. coli* colony was used to inoculate 3 mL LB selection medium in 10 mL reagent glasses, which were incubated overnight at 37 °C under shaking (200 rpm). Cells were sedimented for 5 min at 10,600 x g and resuspended in 200 µL of 50 mM Tris-HCl (pH 7.5) supplemented with 10 mM EDTA and 0.1 mg/mL RNase A and transferred into 2.2 ml Eppendorf tubes. 200 µL of 1 % (w/v) SDS in 200 mM NaOH were added and mixed with the cell suspension by carful inversion of the reaction tube followed by incubation for 5 min at room temperature. For pH neutralisation and SDS precipitation 200 µL of 1.5 M potassium acetate (pH 4.8, adjusted with acetic acid) were added. Cell debris and denatured genomic DNA were separated by centrifugation for 10 min at 18,000 x g and the plasmid containing supernatant was transferred into fresh 1.5 mL reaction tubes. For plasmid precipitation, 750 µL of ice cold isopropanol (100 % (v/v)) were added, mixed by vortexing, incubated at -20 °C for 10 min, and centrifuged at 18,000 x g for 20 min at 4 °C. After discarding of the supernatant, the pellet was washed with 500 µL 70 % (v/v) ethanol followed by centrifugation at 8,600 x g for 5 min at 20 °C. Then, ethanol was removed and the pellet was allowed to air dry (approximately 15 min). For plasmid dissolution, 35 µL of 5 mM Tris-HCl (pH 8.0) were added and incubation for 5 min at 65 °C was conducted to destroy remaining DNases. The obtained plasmids were examined by analytic restriction analysis (2.5.4.1) and gel-electrophoresis of DNA (see 2.5.3.2) and finally stored at -20 °C.

2.5.4.5 Sequencing of DNA

For Sanger sequencing the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit was used (conditions see Tab. 4.). 1.2 µL BigDye® buffer (2.5 x concentrate), 0.8 µL BigDye® reaction mix, 1 µL primer (5 pmol/µL), 100 ng template DNA, and nuclease free water were mixed, reaching a final volume of 5 µL.

Tab. 4. PCR conditions for sequencing reactions
For DNA amplification by PCR the following cycle conditions were used: the elongation time (step 4) was 1 min 15 s, for the first 14 repeats, 1 min 30 s for the next 5 repeats, and 2 min for the final 10 repeats.

Cycle no.	Function	Time [min]	Temperature [°C]	
1	Initial denaturation	01:00	96	
2	Denaturation	00:10	96	 **30 repeats**
3	Primer annealing	00:05	50	
4	Polymerisation (stepwise elongated)	**01:15** 01:30 02:00	60	
5	Pause	∞	10	

The obtained PCR fragments were purified by Sephadex G-50 (equilibrated in H_2O) gel-filtration in a 96-well plate. After application of the PCR reactions, the plates were centrifuged at 950 x g for 5 min at 20 °C. Purified PCR fragments were applied to a Applied Biosystems 3130 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA; Max Plank Institute for Marine Microbiology, Bremen, Germany).

2.5.4.6 PCR amplification of genes of interest

As a first step towards construction of RNAi vectors, genomic DNA fragments containing the entire gene of interest were amplified. Primer pairs were designed using the Gene Runner software version 3.05 (Tab. 1) and obtained from Eurofins MWG GmbH (Ebersberg, Germany). Annealing temperatures were chosen as described in Tab. 5. A proof-reading Taq polymerase protocol (see 2.5.2) was used.

Tab. 5. Primers used for amplification of genomic DNA fragments

Gene fragment	Primer name	Annealing temperatures [°C]	Fragment size [bp]	Sequence (5'→3')
TPS1	TPS1_1S	52	2000	GACCGCTTCTTGTCTGGTATC
	TPS1_1AS			GTGTAAGGAAGAGTTCAAGCG
	TPS1_2S	55	1998	TTAGCTCACGTCGGTACGTTC
	TPS1_2AS			CACCATGCGTAATGCAGGTG
TP	TP_1S	59	2492	GAAGGATCAATGACGGTTG
	TP_1AS			GGGTCATCTGATTAACCCG
	TP_2S	53	2518	GTCTCACTCAAGAAGCCAAG
	TP_2AS			TGTACGAAGGAAGACTCGG
NT	NT_1S	56	2505	GAGATGACTGGCCAAACAGAC
	NT_1AS			ACCAGACTCCCGGACTGATC
	NT_2S	56	2382	GGAAACCGAGGCTACTCATTTC
	NT_2AS			TCTGGTCGCATTTCCATCCTC
GS	GS_1S	55	2516	AATAGACCTACGAACAACACTG
	GS_1AS			CCATGTTATGAGTCACGATG
	GS_2S	55	2504	TCCTACACCGTTGAGGCTC
	GS_2AS			GATTCGATCATAGTATCCGG

All PCR-amplified DNA fragments were sequenced after initial cloning to ensure proper DNA amplification.

2.5.4.7 PCR amplification of genomic DNA fragments and introduction of novel restriction enzyme digestion sites

To allow the construction of inverted repeats for RNAi-induced gene silencing, restriction enzyme recognition sites had to be introduced into genomic DNA fragments (Fig. 13) using a PCR-based strategy (primers see Tab. 6).

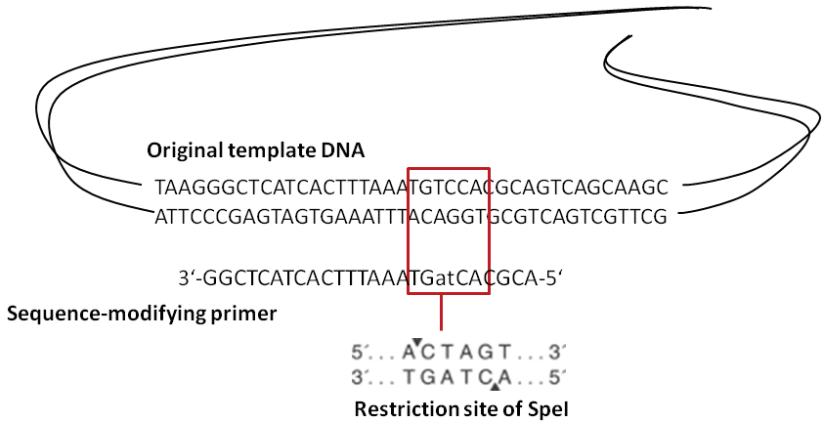


Fig. 13. Introduction of a new endonuclease restriction sites by a PCR-based strategy
The primer for introducing a new restriction enzyme recognition site (*SpeI*, red box) is indicated below the sequence of the template DNA. The *SpeI* site was introduced by exchanging of T to A and C to T (small letters indicate the nucleotides to be exchanged).

Tab. 6. Primers used for DNA fragment amplification for RNAi constructs
For each RNAi construct two DNA fragments for a given gene were obtained by combining always the first primer of the table with the second or third one. Letters in bold highlight modifications of the DNA sequence necessary to introduce endonuclease recognition sites. Listed are also annealing temperatures for PCR reactions and the length of the obtained DNA fragments. Primers were obtained from Eurofins MWG GmbH.

Gene fragment	Primer name	Annealing temperatures [°C]	Fragment size [bp]	Sequence (5'→3')
TPS1	TPS1_ex1_f	56	522	CCTCTCTGGTTTCAAGAAATC
	TPS1_ex1_r_Spe			ACGCA**CtaGTA**AATTTCACTACTCGG
	TPS1_exin1_r_Spe		586	GCCGCA**CTaGtA**ATATCCTACAC
NT	NT_Ex2for	55	266	TCCTACCTTCATGGTTACAACC
	NT_Ex2Spe			CCACCAAC**aCTAgT**ATCGAAGGTC
	NT_Ex2inSpe		321	ATA**ctAGT**ACCCTATGGAACATTAGC
TP	TP_for_ex2	60	295	CGCTCATCATACTGTGGG
	TP_rev_ex2Spe			TCTAACTGTGGA**ACtAGT**CGTAC
	TP_rev_exin2		388	ATGTTGAAGACAATGGGAAC
GS	GS_Ex1for	55	447	ATGTCGGACGATAAACGC
	GS_Ex1Spe			GACTTCA**aCtAGt**AACCACGG
	GS_ExIn1Spe		503	CGT**ACTAGt**ATCGGAAGTACAGGTC

PCR fragments were blunt-end ligated into pJet1.2 (chapter 2.5.4.3). Clones were selected for further purpose in a way that the *ClaI* site of the multiple cloning site of the vector was located: a) at the 3'-end (with regard to mRNA) of the exon containing DNA fragment or b) at the 5'-end (with regard to mRNA) of the exon plus intron containing DNA fragment.

2.5.5 Quantification of gene expression

2.5.5.1 Isolation of total RNA

Total RNA was isolated from 50 mg (dry weight) freeze-dried mycelial powder using the NucleoSpin® RNA Plant kit (MACHEREY-NAGEL GmbH & Co. KG) according to the manufacturers' instructions. RNA isolation included DNase treatment to remove contaminating genomic DNA. Differing from the manufacturers' instructions a 20 % increased DNase amount was used. The RNA was obtained in a final volume of 50 µL DEPC-treated double-distilled H_2O (DEPC-H_2Odd).

2.5.5.2 cDNA synthesis

DEPC-H_2Odd was used in all steps if necessary. cDNA synthesis was performed in 200 µL PCR reaction tubes and all incubation steps (except 0 °C) were carried out in a thermocycler (PEQLAB Biotechnologie GmbH).

For synthesis of first strand cDNA, 200 ng of total RNA was incubated with 100 pmol oligo(dT)18 in a total volume of 12 µL at 65 °C for 5 min followed by 5 min incubation on ice. After brief centrifugation, 4 µL 5 x RT buffer (for reverse transcriptase Revert Aid™ Premium, Fermentas GmbH), 0.5 µL RNase Inhibitor (RiboLock™ (40 U/µL), Fermentas GmbH), 2 µL of DEPC-H_2Odd, 0.5 µL dNTPs (25 mM, Fermentas GmbH), 1 µL RT Revert Aid™ Premium (200 U/µL, Fermentas GmbH) were added. cDNA synthesis was conducted at 50 °C for 30 min and terminated by heat inactivation of RT (85 °C for 5 min). First strand cDNA was shock-frozen in liquid nitrogen and stored at -80 °C.

2.5.5.3 Quantitative RT-PCR (qPCR)

2.5.5.3.1 Primer selection for qPCR

For quantification of mRNA amounts the PCR amplified DNA fragments were chosen such that an exon-intron region of the respective gene, located 5' of the RNAi target, was covered.

Tab. 7. qPCR primers for expression analysis
Primer sequences and length of the PCR products for cDNA and (putative) contaminating genomic DNA are shown for TPS1, NT, TP, GS, and GP.

Gene	*TPS1*	*NT*
Reverse primer (5'→3')	TCTGTACGCCGTGAGTGAC	GTGAGGGTTTCGGGTGGATG
Forward primer (5'→3')	AATTCGGAGAGGATCATAACG	CGGCACCAAAGAAGACCTC
Fragment cDNA [bp]	120	115
Fragment gDNA [bp]	173	169
Gene	*TP*	*GS*
Reverse primer (5'→3')	ATTCTCCAAGGACATCGTC	GGGTATGGAAAGAATGAAAC
Forward primer (5'→3')	ATGTAGGGCTTCAGAGACC	GATAATCGCTCGTGTTGAG
Fragment cDNA [bp]	136	122
Fragment gDNA [bp]	187	179
Gene	*GP*	
Reverse primer (5'→3')	CGAAATCGTCGGTGAGGAG	
Forward primer (5'→3')	AACAGAAATGCCCAGCCTTG	
Fragment cDNA [bp]	132	
Fragment gDNA [bp]	185	

Primer pairs (Tab. 7) were designed using Gene Runner (version 3.05) software. Selected primers were in silico examined for putative cross hybridisation with other genes using BioEdit (version 7.0.9.0) and the current *Laccaria* transcriptome sequence (version 2.0, JGI).

2.5.5.3.2 Quantification of gene expression using 18S rRNA as external standard

The primer pair Lb 18s-2A: 5'-CAGAGCCAGCGAGTTTTTTC-3' and Lb 18s-2B: 5'-GTTTCCGGCTCCCCAAAGC-3', leading to the amplification of a 231 bp DNA fragment, was successfully applied in previous studies for calibration of mRNA content (Dietz, von Bulow et al. 2011) and was thus used in this study, too.

For PCR reaction, 1 µL cDNA solution, 10 µL ABsolute™ QPCR SYBR® Green reaction mixture (Fermentas GmbH), 8.9 µL H_2Odd, and 0.1 µL primer pair mixture (50 mM) were mixed in 96 well plates (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany*)*. A LightCycler®480II system *(*Roche Deutschland Holding GmbH) was used for PCR and detection of the fluorescent signals. Melt curves of the amplified PCR fragments were implemented in the PCR program (Tab. 8) to detect primer dimers and PCR fragments of residual gDNA.

PCR reactions were always performed in duplicates and at least six independent replicates were carried out for any given cDNA.

Tab. 8. Cycle conditions for qPCR
For DNA amplification by PCR the following cycle conditions were used. For signal detection (cycle 5) a slightly increased temperature (79 °C) was used to exclude any primer dimers signal.

Cycle no.	Function	Time [min]	Temperature [°C]	
1	Initial denaturation for activating the polymerase	15:00	95	
2	Denaturation	00:10	95	
3	Primer annealing	00:30	56	**45 repeats**
4	Polymerisation	00:30	72	
5	Camera step	00:02	79	
6	Initial denaturation	00:01	95	
7	Cooling	00:01	60	
8	Melt curve → 0.11 °C/s	Continuous	95	
9	Pause	∞	40	

The Crossing Point (CP) analysis scheme, included in the accessory software, was used for analysis of fluorescent signals. Crossing Points are defined as the PCR-cycle

at which the detected fluorescence rises above the background fluorescence (Rasmussen, Morrison et al. 1998; Pfaffl 2001).

External calibration curves were used to determine the exact template content in every RNA sample. For this purpose, purified PCR fragments of all chosen genes were obtained and the DNA content was quantified photometrically (Nano Drop™ DN1000). Dilutions ranging from 10^{-3} to 10^{-9} were generated and applied to qPCR. Linear regressions were used to calculated slopes of the dilution series. These slopes were further used to calculate the starting template molecule content in the cDNA samples (Pfaffl and Hageleit 2001; Nolan, Hands et al. 2006). The target/reference ratios were used to compare starting target molecule numbers in different samples of total RNA.

2.5.6 Transformation of *Escherichia coli*

2.5.6.1 Generation of transformation competent *E. coli*

Two *E. coli* strains (TOP 10 F‘ and SURE) were used in this work. To receive competent bacterial cells, 3 mL of LB medium (supplemented with kanamycin in case of the SURE strain) were inoculated with a single grown *E. coli* colony and incubated over night at 37 °C under continuous agitation (200 rpm). Thereafter, 50 mL fresh LB medium, in a 100 mL Erlenmeyer flask, were inoculated with the overnight culture resulting in an OD_{600} of almost 0.08 and incubated until an optical density of 0.6 was measurable (shaking at 110 rpm). Bacteria were then transferred into a 50 mL centrifugation tube and centrifugated for 10 min at 4,500 x g and 4 °C. The resulting cell precipitate was resupended in 4 mL of TSB buffer (Chung and Miller 1988) (Transformation and Storage Buffer = LB medium supplemented with 10 % (w/v) PEG 3350, 5 % (v/v) DMSO, and 20 mM $MgSO_4$). Each 200 µL of resuspended, competent cells were filled into 1.5 mL reaction tubes and frozen in liquid nitrogen for long term storage at -80 °C.

2.5.6.2 Transformation of competent *E. coli*

Aliquots of competent *E. coli* cells were thawed on ice and transformation was carried out by addition of 100 µL DNA solution consisting of 20 µL KCM solution (100 mM KCl, 30 mM $CaCl_2$, 50 mM $MgCl_2$), up to 20 µL of a ligation reaction or 100 ng of plasmid, and H_2Odd to reach the total of 100 µL. Competent cells and DNA solution were carefully mixed by pipetting and incubated on ice for further 20 min, followed by a 2 min heat shock at 42 °C and subsequent addition of 500 µL LB medium. The cell suspension was incubated at 37 °C for 1 hour under slow agitation, followed by cell

sedimentation at 950 x g for 5 min and removal of 700 µL of the supernatant. After resuspension, cells were streaked onto LB plates (supplemented with appropriate antibiotics) using a sterile Drigalski spatula and incubated over night at 37 °C.

2.5.7 Transformation of *Agrobacterium tumefaciens*

2.5.7.1 Generation of transformation competent *A. tumefaciens*

To receive competent agrobacterial cells, 10 mL of King's B media, supplemented with strain-specific antibiotics, were inoculated with a single colony of a particular *A. tumefaciens* strain. The inoculum was grown for 2 days at 28 °C under agitation (200 rpm) and used as inoculum for 200 mL selective King's B medium (in a 500 mL Erlenmeyer flask). Cells were incubated at 28 °C under agitation (agitation at 110 rpm) until an OD_{600} of 0.5 was reached. Agrobacteria were sedimented by centrifugation at 3000 x g for 5 min and 20 °C. The supernatant was discarded and cells were resuspended in 100 mL of 0.15 M NaCl. After centrifugation at 3000 x g for 5 min and 20°C the pellet was dissolved in 10 mL 20 mM $CaCl_2$. 200 µL aliquots of the competent cells were quick-frozen in liquid nitrogen and stored at -80 °C.

2.5.7.2 Transformation of competent *A. tumefaciens*

Competent agrobacteria were carefully thawed on ice for a minimum of 1 hour. 200 ng of plasmid DNA were added and cells were incubated on ice for further 10 min. Bacteria were frozen in liquid nitrogen for 5 min and a heat shock was applied by incubation at 37 °C for 5 min. For cell regeneration 800 µL of King's B medium were added and cells were incubated at 28 °C for 3-4 h. Cells were sedimented by centrifugation at 3000 x g for 5 min, 900 µL of the supernatant were removed and cells were resuspended in the remaining. Cells were plated onto petri dishes containing selective King's B medium and incubated for 2-3 days at 28 °C until colonies were formed.

2.5.8 Transformation of *Laccaria bicolor*

Two techniques for transformation of the ectomycorrhizal fungus *Laccaria bicolor* were described in literature: protoplast (Barrett, Dixon et al. 1990) and *Agrobacterium*-based (Kemppainen, Circosta et al. 2005; Kemppainen, Duplessis et al. 2009; Kemppainen and Pardo 2011). As batches of enzymes, necessary for cell wall digestion, differ strongly in their capability in efficient protoplast formation and transformation efficiency can be rather low when using this technique, *Agrobacterium*-based transformation was

chosen in this study. However, transformation was not successful with the fungal strains used in this work, when applying protocols published for *Laccaria* (Kemppainen, Circosta et al. 2005; Kemppainen, Duplessis et al. 2009; Kemppainen and Pardo 2011). Therefore, a modified protocol, based on *Agaricus bisporus* transformation (Chen, Stone et al. 2000), was developed for successful transformation of *L. bicolor* (see below for final protocol).

Both organisms were pre-grown in parallel approximately 10 days before transformation.

A single agrobacterial colony, grown from a fresh transformation, was used to inoculate 3 mL of selective King's B medium. After 2 days of incubation at 28 °C under agitation (200 rpm), the culture was stored in a fridge (4 °C) for up to few days. One day prior to transformation, 100 µL of the bacterial suspension was transferred into 50 mL selective King's B medium (in a 250 mL Erlenmeyer flask) and cells were incubated over night at 28 °C under agitation (120 rpm) until an OD_{600} of approximately 0.8 was reached. Agrobacterial suspension was added to 50 mL selective King's B medium in a 100 mL Erlenmeyer flask to obtain an OD_{600} of 0.3. To induce *A. tumefaciens* virulence gene expression, 200 µM acetosyringone were added and the culture was incubated for 6 h at 24 °C and slow agitation (80 rpm). Bacteria were harvested by centrifugation at 3000 x g for 10 min at 20 °C and resuspended in 10 mM $MgSO_4$ (supplemented with 0.05 % (v/v) Silwett L-77) to reach a final density between 3.5 and 4.5 OD_{600}.

For pre-cultivation of *Laccaria bicolor* strains (S238N-H70 and S238N-H82) cellophane membrane-covered MMN plates (supplemented with 25 mM glucose) were inoculated with small agar blocks (2 x 3 mm) obtained from the growing mycelial front of a 2 week old petri dish culture (Fig. 14).

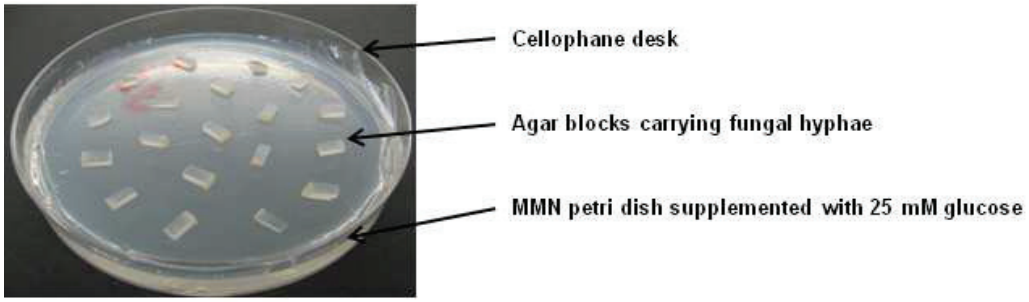


Fig. 14. Pre-cultivation of *Laccaria* for transformation
Petri dishes, containing cellophane covered MMN medium supplemented with 25 mM glucose, were inoculated with 15-20 hyphae-carrying agar blocks. Hyphal grow was allowed for approximately 10 days at 18 °C in darkness.

Petri dishes were incubated at 18 °C in darkness for 10 days. For fungal transformation colonies containing cellophane membranes were transferred onto fresh MMN agar plates (18 g/L Serva Kobe 1 agar) supplemented with 300 µM 3, 5-dimethoxy-4-hydroxy-acetophenone (acetosyringone), 10 mM MES-KOH buffer (pH 5.2), 1 mM betaine, 0.5 % (w/v) glucose, 0.5 % (w/v) glycerol, and 0.5 % (w/v) sucrose. 1 mL of concentrated virulent bacteria was slowly dropped onto fungal mycelia (Fig. 15).

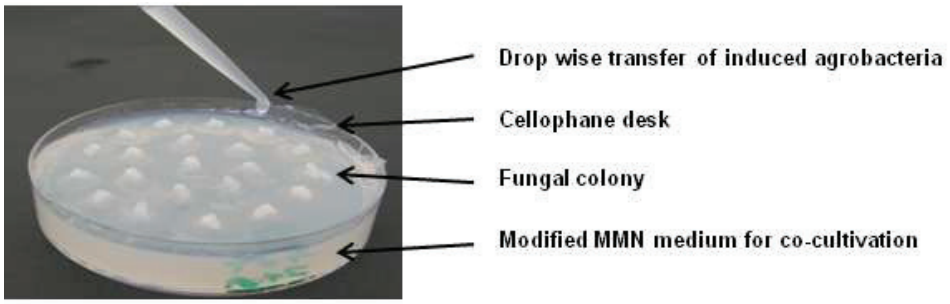


Fig. 15. Inoculation of *Laccaria bicolor* with transgenic *Agrobacterium tumefaciens*
1 mL of concentrated virulent agrobacteria was distributed drop wise onto the fungal hyphae covering the cellophane desk.

The co-cultivation was conducted for 5 days at 24 °C in darkness to allow T-DNA transfer into fungi and hygromycin resistance gene expression. The mycelia covered cellophane membranes were then transferred onto a selective MMN agar plates (supplemented with hygromycin (500 µg/mL), cefatoxim (250 µg/mL) and tetracycline (100 µg/mL) and incubated at 18 °C in darkness (tetracycline is light sensitive and will be degraded otherwise) for about 8 weeks. If the transformation was successful, small hygromycin resistant fungal colonies were formed. These colonies were excised with a sterile scalpel and transferred onto fresh MMN petri dishes (containing 300 µg/mL hygromycin). Frequently sectoral fungal growth was visible after 2-3 weeks of growth at 18 °C in darkness, indicating two or even more different transformed fungi in one colony. If this occurred, a small part of the hyphal front of the colony was excised with a sterile scalpel and transferred onto fresh MMN petri dishes (containing 300 µg/mL hygromycin). Such transfer of small sectors of the mycelial growth front was performed until a homogenously round shaped colony, without any kind of sectoral growth, was obtained. For evaluation of transformation efficiency the number of achieved transgenic colonies was divided by the overall number of fungal colonies used for transformation.

2.5.9 Fungal growth assays

Two assays were performed to characterise the impact of genetic background (monokaryotic *Laccaria* strains) or transgene expression on fungal growth behaviour.
For determination of the maximal growth speed of the hyphal front MMN agar plates, supplemented with 25 mM glucose and covered with a cellophane membrane, were inoculated with a small agar block (3 x 3 mm) containing the mycelial front of a growing fungal colony. After fungal pre-growth in a climate chamber at 18 °C in the dark, necessary to allow mycelia an initial colonisation of the new substrate, the agar plates were further incubated at 18 °C (for temperature stress experiments 12, 18, 22, 24, 26, 28, or 30 °C were used) in the dark for up to 4 weeks and growth of the mycelial front was recorded every 7th day. The experiment was stopped when the fungal colony covered about 2/3 of the agar plate. The increase in diameter of the fungal colony was determined for all four growth directions according to Fig. 16. Increase in colony radius of 12-16 replicates was followed in all four growth directions over time and used to calculate the maximal hyphal growth speed per day.

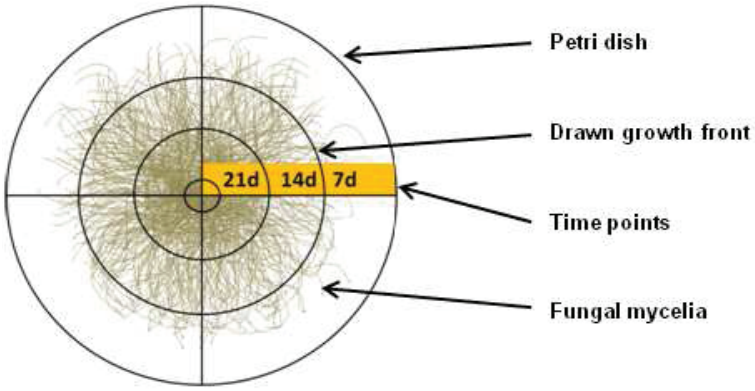


Fig. 16. Scheme of fungal colony harvesting
Petri dishes containing MMN agar supplemented with 25 mM glucose and covered with a cellophane membrane were inoculated with a 3 x 3 mm agar block from the mycelial front of a growing fungal colony. After 10 days of growth the increase in diameter of the fungal colony was followed for about 3 weeks and was determined for all four growth directions over this growth period. When the colony had occupied about 2/3 of the agar plate, it was divided into three parts, the outermost area (containing the youngest hyphae, maximal **7 d**ays old), the middle section (hyphal age between 8 and **14 d**ays), and the oldest part (mycelial age 15 to **21 d**ays) using a sterile scalpel. The central inoculum area was wasted. Mycelia were harvested, transferred into 1.5 mL micro-reaction tubes, snap-frozen in liquid nitrogen and lyophilised.

For determination of the mycelial density (which reflects hyphal branching), mycelia from the same agar plates used for determination of maximal growth speed were divided into three parts (Fig. 16): the outermost area (containing hyphae that were maximal 7 days old), the middle section (hyphal age between 8 and 14 days), and the

oldest part (mycelial age 15 to 21 days) using a sterile scalpel. Mycelia were harvested in 1.5 mL micro-reaction tubes, frozen in liquid nitrogen and stored at -80°C until further processing. Samples were lyophilised at -20 °C for a period of 4 weeks by applying a vacuum of 5 x 10^{-3} mbar. The weight of the mycelial samples was afterwards determined in a laboratory with adjusted relative humidity of 40 % at 17 °C.

2.5.10 Formation of dikaryotic mycelia from monokaryotic *Laccaria* strains

For ectomycorrhizal symbiosis dikaryons are needed (Martin and Selosse 2008; Smith and Read 2008; Kemppainen, Duplessis et al. 2009; Plett, Kemppainen et al. 2011). Therefore, transformants of selected fusion compatible (Prof. Dr. F. Le Tacon, INRA, France, personal information) monokaryons (S238N-H70 x S238N-H82) were grown together on agar plates. For fusion, small agar blocks (2 x 3 mm) containing inoculums of compatible transformants were placed face to face onto MMN agar plates supplemented with 25 mM glucose and covered by a cellophane membrane (Fig. 17).

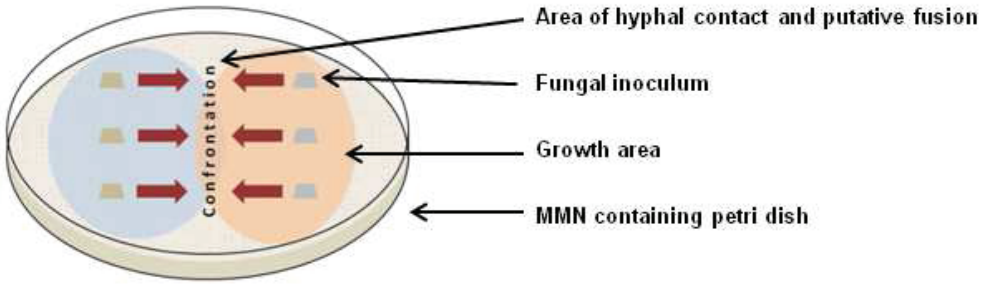


Fig. 17. Initiated fusion of compatible fungal isolates
Compatible fungal strains grew on a cellophane desk-covered MMN agar dish for approximately 30 days. Colonies are indicated as small trapezes. The area of hyphal overlay was transferred to selective conditions to select for dikaryons. This “Confrontation” areal is highlighted by overlap of both growth areas (blue and orange) as further indicated by the red arrows.

After 30 days of incubation at 18 °C in darkness a contact front of hyphae became visible (see Fig. 18). Small stripes (20 x 3 mm) of the confrontation area were excised with a sterile scalpel (highlighted as blue squares in Fig. 18) and transferred onto MMN agar plates (supplemented with 25 mM glucose, 25 mM $(NH_4)_2HPO_4$, and 200 µg/mL hygromycin). Under this condition, dikaryons are expected to grow faster than the respective monokaryons.

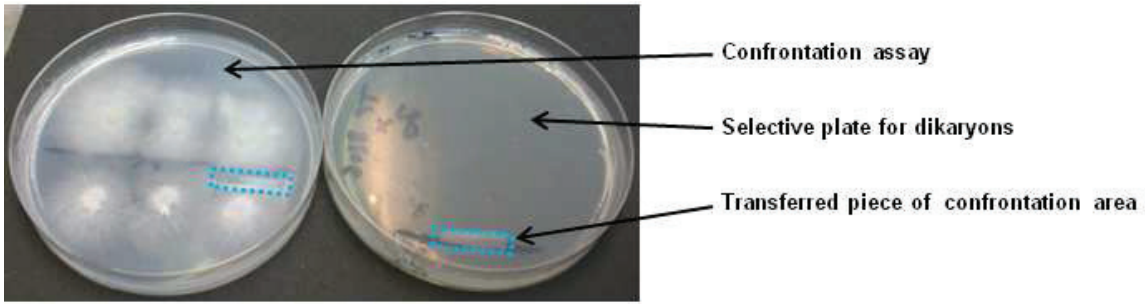


Fig. 18. Transfer of putatively dikaryotic mycelia onto selective agar medium
After 30 days of growth, pieces of the confrontation zone (left petri dish) were excised and transferred to dikaryon-selective medium (petri dish on right site).

After further 20-30 days of growth at 18 °C in darkness, small pieces of the mycelial front were excised and transferred to fresh selection agar plates. This transfer of small parts of the mycelial front was repeated until a homogenous round shape colony without any sectors was formed.

2.5.10.1 Microscopic analysis of fused fungal strains

The final proof for dikaryotic mycelia was performed by cell wall staining of hyphae with Calcofluor White (Monheit, Cowan et al. 1984; Harrington and Hageage 2003; Martín-Udíroz, Madrid et al. 2004), to inspect hyphae for closed clamp connections and septa formation (Raudaskoski and Kothe 2010). For this task, the chitin- as well as cellulose-specific dye was applied to stain the produced potential dikaryons, which were pre-grown on cellophane desk-covered MMN medium. One drop of 0.05 % (v/v) Triton X-100 was pipetted onto the hyphal front of a growing fungal colony to detach the mycelia from the cellophane membrane. Small pieces of the mycelial front were transferred into a drop of 10 % (w/v) potassium hydroxide on a glass slide and 30 µL of Calcofluor White M2R staining solution (Sigma-Aldrich Corporation, St.Louis (MO), United States of America) were added prior to cover slip addition. After incubation at 20 °C for 10 min samples were inspected at 400 x magnification with a Leica DMRB microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) under UV light (LEj LQ-HXP 120, Leistungselektronik JENA GmbH, Jena, Germany) using the A filterblock (excitation 340-380 nm, emission > 430 nm). Images were taken using a Leica DFC425C camera and the LAS 2.0 (Leica Application Suite) software package.

3 Results

3.1 Selection of genes for RNAi suppression

Based on gene expression studies and elevated trehalose content in *Amanita muscaria* ectomycorrhiza, trehalose metabolism is expected to be central for efficient fungal sink formation in symbiosis (Lopez, Manner et al. 2007). The aim of this work was to manipulate gene expression of selected proteins central for trehalose and glycogen turnover. *L. bicolor* was chosen as target organism because of its sequenced genome (Martin, Aerts et al. 2008) and previously published reports on manipulation of fungal gene expression (Kemppainen, Duplessis et al. 2009; Plett, Kemppainen et al. 2011). Genes putatively involved in storage carbohydrate metabolism of *Laccaria bicolor* S238N-H82 were annotated in previous work (Deveau, Kohler et al. 2008; Martin, Aerts et al. 2008; Martin and Selosse 2008). This annotation is, however, based on an early version (1.0) of the *L. bicolor* genome. Until then, extended resequencing of the genome and large scale gene expression studies were carried out. The published scheme of trehalose and glycogen metabolism in *Laccaria bicolor* S238N-H82 (Deveau, Kohler et al. 2008) was updated using actual (version 2.0) genome sequence data in this work. The predicted protein models and their corresponding genes were used for BLAST (Altschul, Madden et al. 1997) searches in different online databases to discover the most potent function. *Laccaria* sequences together with proteins from other organisms were used for phylogeny analysis to identify functional protein families. The results for trehalose metabolism are summarised in Tab. 9.

Tab. 9. Putative enzymes involved in trehalose metabolism of *Laccaria bicolor*
Displayed are the JGI protein IDs of predicted proteins of trehalose metabolism of *Laccaria bicolor*, identified using the KOGG genome browser or BLAST analysis of the *Laccaria* genome database (version 2.0), the length of the predicted protein, and the localisations of the corresponding genes within the genome. Protein IDs in bold indicate the proteins selected for further alignment-analysis and RNAi approaches. For some enzymes more than one ID was identified in the database.

Function	Protein ID/predicted scaffold	Amino acids [aa]	Localisation
α,α-trehalose-6-phosphate synthase TPS1	**234950**/ Lacbi1.e_gwh1.11.143.1	511	LG_3:3617555-3619301
Acid trehalase	245054/ Lacbi1.e_gww1.3.81.1	662	LG_4:906994-909999
Hypothetical protein	256049/ Lacbi1.e_gww1.71.22.1	309	LG_9:779853-780930
Neutral trehalase (α,α-trehalase)	**297216**/ Lacbi1.eu2.Lbscf0011g0373	775	LG_3:3517513-3520263

	0		
Trehalose-6-phosphate phosphatase TPS2	306794/ Lacbi1.eu2.Lbscf0032g00660	907	LG_6:880287-884039
Regulatory subunit of TPS-complex - TSL1/TPS3	318750/248803 Lacbi1.eu2.Lbscf0009g01100	972	LG_8:1029811-1033334
Trehalose phosphorylase TP	**315967**/708481 (new 293441) Lacbi1.estExt_fgenesh2_pg.C_60442	742	scaffold_11:1801410-1804461
Candidate a,a-trehalose-6-phosphate synthase	318760/ Lacbi1.eu2.Lbscf0009g01200	99	LG_8:1054827-1055126
Putative trehalase (not curated)	477291/255655 fgenesh1_pm.LG_1_#_8	292	LG_1:44669-45992
Trehalose-6-phosphate synthase component TPS1 and related subunits (not curated)	620522/ e_gw1.22.393.1	115	scaffold_22: 126732-127405
Trehalose-6-phosphate synthase component TPS1 and related subunits (not curated)	620603/ e_gw1.22.268.1	115	scaffold_22:267522-268190

Accordingly, single copy genes coding for trehalose-6-phosphate synthase (TPS1, protein ID 234950), trehalose-6-phosphate phosphatase (TPS2, protein ID 306794), TPS-complex regulatory subunit (TSL1/TPS3, protein ID 318750), and trehalose phosphorylase (TP, protein ID 293441) could be confirmed. However, rescreening of the current version of the *Laccaria* genome by using the BLAST algorithm revealed three additional truncated TPS1-like proteins (IDs 318760, 620522, and 620603) with potentially regulatory functions, because all three grouped together with known TSL1 and TPS3 proteins. In contrast, only one neutral trehalase (NT, protein ID 297216) gene was annotated (Deveau, Kohler et al. 2008). Rescreening of the actual version, however, revealed two additional genes coding for trehalase-like proteins within the genome (IDs 477291 and 256049), see dendrogram in Fig. 19 (*Laccaria*-specific proteins are indicated by the blue background). Furthermore, a gene coding for a putative trehalose transporter (JGI-protein ID 639283) was identified, which also had not been previously annotated. Finally, the calculations confirmed the function of protein 245054 as trehalase, proposed by Deveau and co-workers (2008) to be an AT that, however, now did not group into the acid trehalase branch of the approved acid trehalases of some ascomycetes (AT branch on left side of Fig. 19).

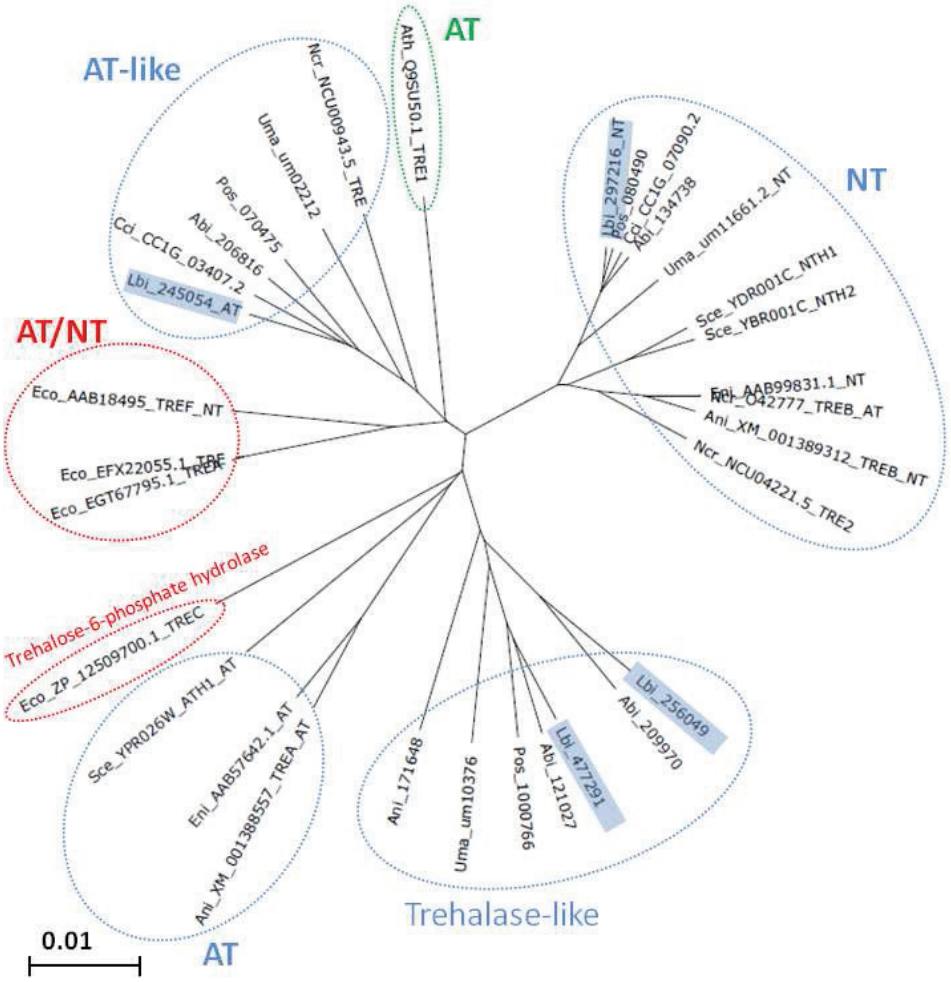


Fig. 19. Dendrogram of known trehalases and selected trehalase-like proteins
Amino acid sequences of neutral trehalases (NT), acid trehalases (AT), and selected trehalase-like proteins were obtained from KOG databases and BLAST searches. Green encircled proteins are from *Arabidopsis thaliana*, red highlighted members from *Escherichia coli*, and all proteins of fungal origin are encircled in blue. Additionally, the *Laccaria*-specific sequences are marked by blue bars. The branch length in the tree reflects replacements per amino acid site (see scale). Determination of protein families was performed on the basis of proteins with known functions from *Arabidopsis thaliana* (apoplastic TRE1 (Frison, Parrou et al. 2007)), *Saccharomyces cerevisiae* (neutral trehalases NTH1, NTH2, acid trehalase ATH1 (Londesborough and Varimo 1984; Destruelle, Holzer et al. 1995; Nwaka, Mechler et al. 1995; Nwaka, Mechler et al. 1996; Garre and Matallana 2009)), *Emericella nidulans* (neutral trehalase TREB (D'Enfert, Bonini et al. 1999)), *Neurospora crassa* (neutral trehalases TREB/TRE2 (D'Enfert, Bonini et al. 1999)), and *Escherichia coli* (periplasmic trehalase TREA (Horlacher, Uhland et al. 1996; Uhland, Mondigler et al. 2000) and neutral TREF (Horlacher, Uhland et al. 1996)). Trehalose-6-phosphate hydrolase (TREC (Rimmele and Boos 1994) of *E. coli* was included as an out-group. Two novel trehalases-like *Laccaria* proteins (IDs 256049 and 477291), observed in this work, are included,

Based on the known interconnections of glycogen and trehalose metabolism, the glycogen metabolism of *Laccaria* was investigated, too. Thus, different *Saccharomyces* (www.yeastgenome.org) derived proteins, involved in glycogen biosynthesis and degradation, were introduced for further BLAST researches. As *Saccharomyces*

proteins GSY1 and GSY2 (the two glycogen synthases) disclosed only a single *GS* gene (ID 189159) in *Laccaria*. Moreover, using the yeast glycogen branching enzyme GLC3, again a single copy gene was obtained (protein ID 292800). BLAST approaches with both *Saccharomyces* glycogenin proteins (GLG1 and GLG2) were sufficient to identify a single encoded glycogenin (GSI) (ID 298612). As homologue of yeast glycogen phosphorylase GPH1, the protein ID 307719 was identified (GP). However, three *Laccaria* proteins (IDs 302750, 304819, and 679192) were detected as *Saccharomyces* GDB1-like (debranching enzyme), of which especially the first protein shared highest homology (ID 302750). As homologue of yeast glycogen degrading glucoamylase SGA1, a single encoded protein (ID 309096) was discovered in the *Laccaria* database. The *Laccaria*-specific proteins GS, GP, and GSI were published in 2008 as major components of the glycogen metabolism (Deveau, Kohler et al. 2008) that were confirmed in this research. Furthermore, the GS (protein ID 189159) was introduced for the RNAi attempts to be able to identify a connection to the trehalose metabolism *in vivo*. In Tab. 10, the obtained glycogen-dependent *Laccaria* proteins are listed.

Tab. 10. Putative enzymes involved in glycogen metabolism of *Laccaria bicolor*
Displayed are the JGI-predicted *Laccaria bicolor* proteins (and IDs) for glycogen metabolism, identified using the KOGG genome browser or BLAST analysis of the *Laccaria* genome database (version 2.0). Furthermore, the length of the predicted proteins and the corresponding genome localisations are listed. For some enzymes more than one ID was identified in the database.

Function	Protein ID/predicted scaffold	Amino acids [aa]	Localisation
Glycogen (starch) synthase GS	189159/ Lacbi1.estExt_GeneWisePlus_worm.C_50367	741	scaffold_12:807334-810064
Glycogen phosphorylase GP	307719/ Lacbi1.eu2.Lbscf0036g01670	881	LG_8:455139-459828
Glycogen synthase initiator GSI (glycogenin)	298612/ Lacbi1.estExt_fgenesh2_pg.C_10372	1027	LG_1:5565475-5569031
Alpha amylase (glycogen debranching enzyme)	302750/ Lacbi1.eu2.Lbscf0022g00400	1594	scaffold_13:1292478-1297881
Alpha amylase (glycogen debranching enzyme)	304819/304818 Lacbi1.eu2.Lbscf0029g01030	115	LG_4:3032872-3033328
Alpha amylase (glycogen debranching enzyme)	679192/ fgenesh3_kg.LG_1_#_96_#_304819	115	LG_1:284012-284468
Predicted starch-binding protein	309096/ Lacbi1.eu2.Lbscf0003g07350	638	LG_4:456677-459063
1,4-alpha-glucan branching enzyme/starch branching	292800/ Lacbi1.estExt_fgenesh2_pg.C	680	LG_3:1767945-1770570

enzyme II	_40385		

Moreover, to prove the target specificity of RNAi-introduced gDNA fragments, intense nucleotide-BLAST analysis were performed. For chosen 522 bp TPS1-RNAi exon fragment only two genes, with coverage of less than 18 bp, were revealed. The corresponding IDs are 327769 (an unknown protein) and 290773 (phosphotransferase). Same was conducted for selected NT-specific exon fragment of 266 bp with complementary regions of ≤ 18 bp in genes with protein IDs 311426 (rRNA maturation protein Nop14-like family), 313794 (uncharacterised protein), 150801 (helicase), 185727 (copper monoxygenase), 323940 (uncharacterised protein), 294799 (protein kinase), and 313271 (Ca^{2+}-dependent cystein protease). The TP-specific exon fragment of 295 bp contained a 17 bp fragment further located in the coding genes for proteins with IDs 332807 (uncharacterised protein) and 310231 (unknown protein). Finally, the exon fragments of 447 bp for GS-RNAi obtained complementary fragments of less than 18 bp in genes coding for proteins with ID 317283 (uncharacterised protein), 298009 (uncharacterised protein), and 330867 (uncharacterised protein).
Finally, the selected RNAi-exon fragments were further tested for cross-targeting the mRNAs of the other trehalose- and glycogen-metabolic proteins, but no significant coverage was detected. Therefore all RNAi-inducing fragments were determined as highly gene-specific without targeting other mRNAs.

3.2 Identification of potential MSN-like factors

Central part of yeast response to different kind of stresses (nutrient starvation, chemical treatment, elevated temperature) is a signalling cascade leading to transcriptional adaptation of trehalose and glycogen metabolism. Gene regulation is modulated by two transcription factors binding to so-called STRE (stress responsive) elements in the promotor regions of respective genes (Winderickx, de Winde et al. 1996; Gorner, Durchschlag et al. 1998; Thevelein and de Winde 1999; Sunnarborg, Miller et al. 2001). STRE elements in yeast are characterised by the sequence 5'-CCCCT-3'. To address the question whether this type of regulation might be conserved in *Laccaria*, putative promotor regions (2000 bp upstream of the start codon) of five genes selected for expression studies were subjected to in silico analysis for STRE elements using DNASTAR Lasergene® Seqbuilder version 7.1.0 and BioEdit version 7.0.9.0 (see Tab. 1). Accordingly, five putative STRE elements were found for *TPS1* promotor, two for *NT*, and three elements each for *TP* and *GS*, while *GP* revealed eight putative STRE elements (Tab. 11).

Tab. 11. Putative STRE elements in the promotor regions of *TPS1*, *NT*, *TP*, *GS*, and *GP* genes
Shown are the positions of STRE elements, encoded in the indicated gene-specific promotors (distance [bp] upstream of the start codon).

STRE sequence	*TPS1*	*NT*	*TP*	*GS*	*GP*
5'-CCCCT-3'	109-113 140-144 1396-1400	---	143-147	1-5	1530-1534
5'-AGGGG-3'	349-353 762-766	688-692 1962-1966	125-129 1239-1243	467-471 1862-1866	1090-1094 1100-1104 1108-1112 1163-1167 1259-1263 1455-1459 1542-1546

Furthermore, as the transcription factors (MSN2 and MSN4) that bind to STRE elements are known in yeast (Martinez-Pastor, Marchler et al. 1996), a screen for *Laccaria bicolor* homologues was performed using a protein BLAST search strategy (Tab. 12).

Tab. 12. Putative *Laccaria* transcription factor homologues to yeast MSN2 and MSN4
Protein IDs of MSN2- and MSN4-like proteins of *Laccaria bicolor* are listed. Numbers in bold indicate the proteins with highest homology to their respective yeast counterparts. Both *Saccharomyces* proteins were picked from www.yeastgenome.org.

Saccharomyces cerevisiae S288C	*Laccaria bicolor* 238N-H82
MSN2	393192, 681767, **308011**, **301563**, 308583, **607158**
MSN4	681767, 393192, **301563**, **607158**

The yeast STRE element binding proteins belong to the zinc finger transcription factor gene family (Martinez-Pastor, Marchler et al. 1996). The typical zinc finger binding motif of yeast (Sc) MSN factors is well conserved in the putative *Laccaria* (Lb) proteins (Fig. 20).

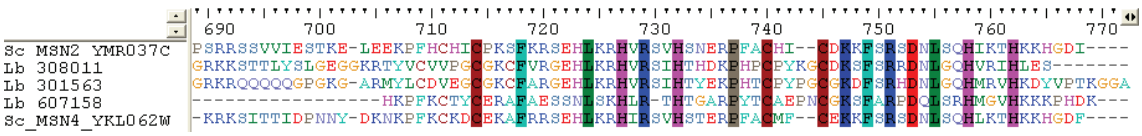


Fig. 20. Zinc finger binding motifs of three putative *Laccaria bicolor* MSN homologues
Displayed is an alignment of the deduced protein sequence of the three most conserved *Laccaria bicolor* MSN-like factors. Identical amino acids are highlighted with same colour.

3.3 Growth behaviour of *Laccaria bicolor* wild type mycelia

For fungal transformation, but also for later phenotyping of transformants, growth behaviours of fungal wild type hyphae on agar plates were determined first, according to chapter 2.5.9. The maximal growth of the hyphal front was used for growth quantification and two parameters, temperature (for monokaryons) and carbohydrate nutrition (all strains), were investigated first (Fig. 21 and 23).

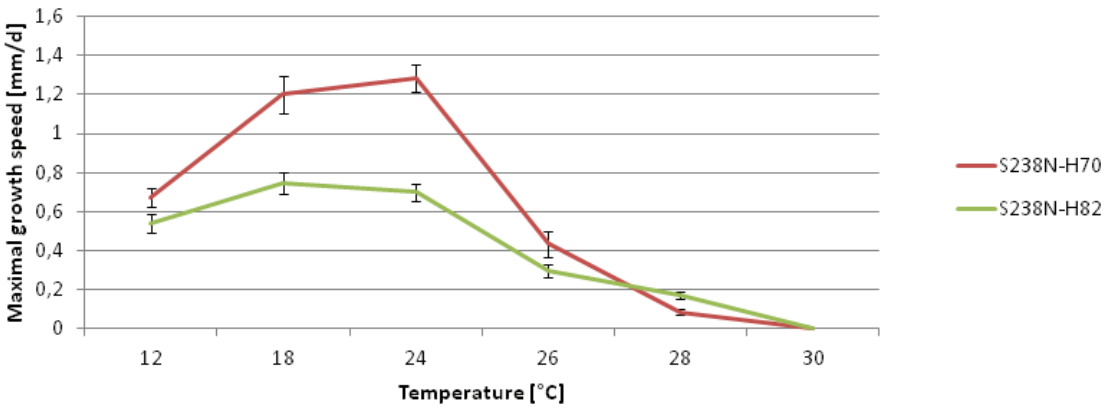


Fig. 21. Temperature-dependent maximal growth speed of monokaryotic *Laccaria* hyphae
MMN-supplemented petri dishes containing 25 mM glucose were inoculated with fungal mycelium. Every 7th day, fungal growth was measured and used to calculate the maximal hyphal growth speed (y-axis) at a given temperature (x-axis). Established monokaryotic *Laccaria bicolor* colonies were exposed to temperatures between 12 and 30 °C.

Temperatures between 18 and 24 °C turned out to be optimal for both *Laccaria* strains. However, hyphae with S238N-H70 background revealed a maximal growth speed of around 1.2 mm/day while those with S238N-H82 background grew much slower (around 0.7 mm/day). An increase in temperature of only 2 °C (24 to 26 °C), resulted in a strong decrease of fungal growth (60 % for S238N-H70 and 50 % for S238N-H82), indicating a temperature of 24 °C to be at the border to heat stress. Fungal growth was hardly visible at 28 °C and colony growth started to become sectoral (especially for H70, see Fig. 22). However, fungal growth was completely abolished at 30 °C. In contrast to temperature increase, a reduction by 6 °C (18 to 12 °C) resulted in retardation in fungal growth speed only by 20 % (S238N-H82) up to 50 % (S238N-H70).

Fig. 22. Initiated sectoral grow of a colony of strain S238N-H70 at 28 °C

Compared to temperature, carbohydrate nutrition (glucose concentrations between 0.25 and 100 mM) had only a minor impact on the maximal growth speed of hyphae for all investigated *Laccaria* strains (Fig. 23).

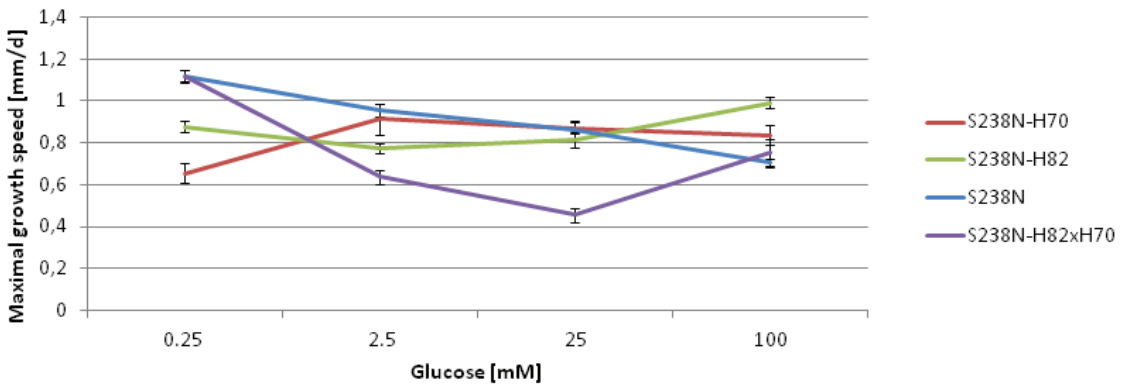


Fig. 23. Dependency of the maximal growth speed of *Laccaria* strains on glucose concentration
MMN plates supplemented with different glucose concentrations were inoculated with agar plugs containing fungal mycelium. Every 7th day, fungal growth was measured and used to calculate the maximal hyphal growth speed (y-axis) at a given glucose concentration (x-axis) of 0.25 mM, 2.5 mM, 25 mM, or 100 mM.

Prior to fungal transformation and dikaryon formation, growth behaviour of the provided two monokaryotic (S238N-H70, S238N-H82) and of two dikaryotic (S238N, S238N-H82xH70) *Laccaria bicolor* strains, on MMN agar plates containing different ammonium phosphate- as well as antibiotic-concentrations, was investigated (Fig. 24 and 25).

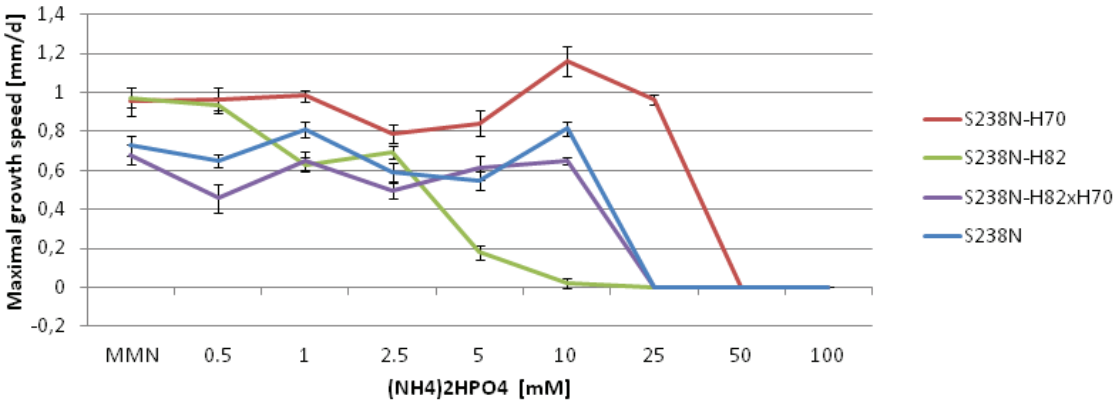


Fig. 24. Impact of $(NH_4)_2HPO_4$ content on *Laccaria bicolor* hyphal growth speed
MMN plates with different $(NH_4)_2HPO_4$ concentrations were inoculated with *L. bicolor* mycelium and the colony diameter was followed over time. The maximal growth speed (y-axis) was calculated and drawn against the corresponding ammonium phosphate concentration (x-axis). Final concentration of 0.5 mM, 1 mM, 2.5 mM, 5 mM, 10 mM, 25 mM, 50 mM, and 100 mM were investigated.

The monokaryotic strain H82 tend out to be highly sensitive to elevated ammonium phosphate concentrations, compared to all other strains. As mycelial growth of H82 decreased already at ammonium phosphate concentrations above 2.5 mM and stopped completely at 10 mM, while all other strains were not affected. Furthermore, all strains except H82 displayed an intense airborne mycelium and a strong lilac colour at elevated ammonium phosphate concentrations that was particularly generated by monokaryon H70. Both tested dikaryons behaved in a similar manner.

To allow selection for transgenic *L. bicolor* hyphae, four antibiotics (BASTA, hygromycin, carboxin, and kanamycin) that can be used for dominant selection, were tested. In Fig. 25 the impact of increasing antibiotic concentrations on fungal growth is shown.

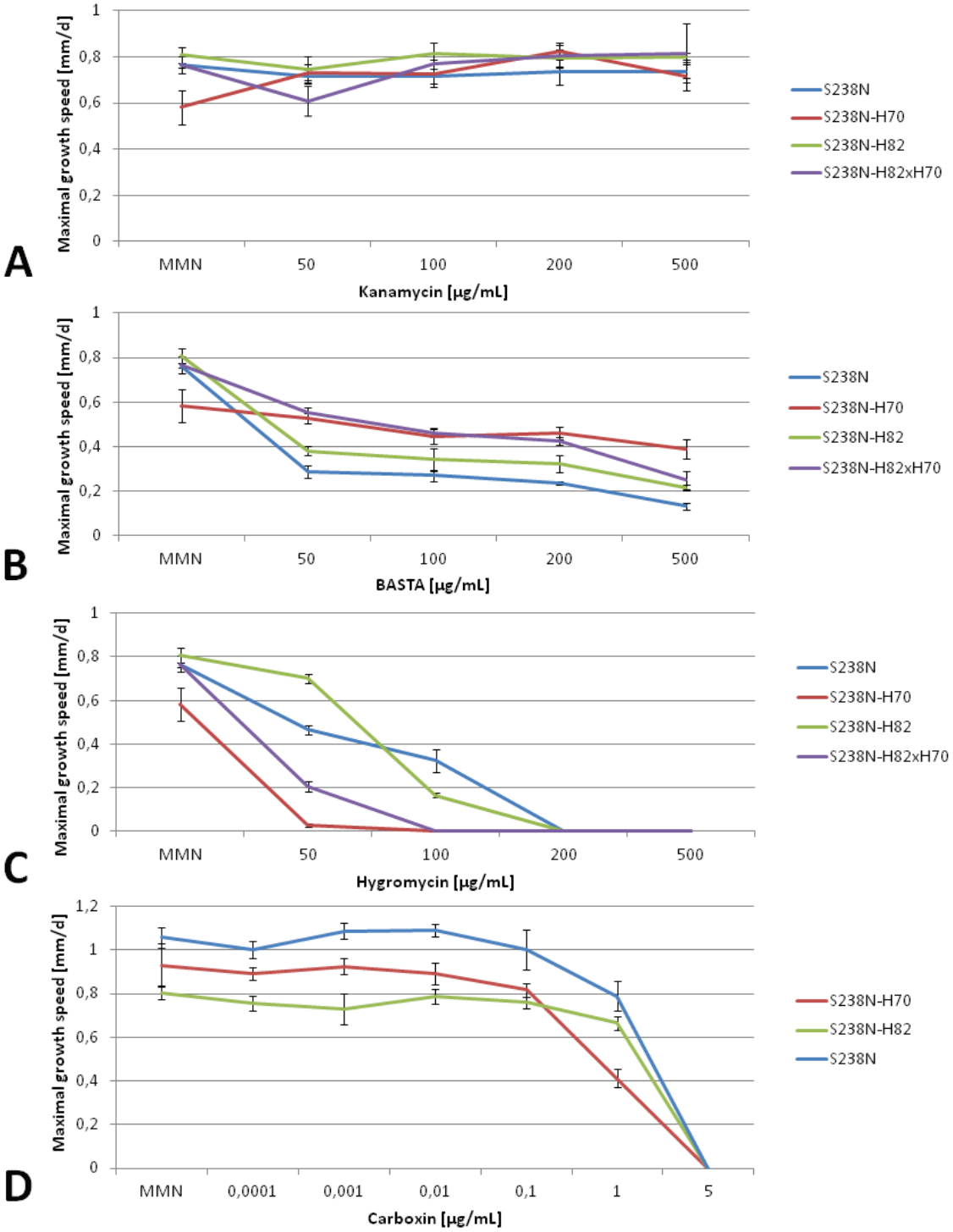


Fig. 25. Inhibition of *Laccaria bicolor* growth by increasing antibiotic concentrations
MMN petri dishes were supplemented with different antibiotics in several concentrations and inoculated with *L. bicolor* mycelia. The colony diameter was followed over time. The maximal growth speed (y-axis) was calculated and drawn against the corresponding antibiotic concentration (x-axis). Two mono- and two dikaryotic fungal strains were investigated for their behaviour on kanamycin (A), BASTA (B), and hygromycin (C), while S238N was the only dikaryon tested for carboxin (D) toxicity.

Kanamycin (Fig. 25 A) was not suitable to inhibit *Laccaria* growth, while the presence of 200 µg/mL hygromycin (Fig. 25 C) or 5 µg/mL carboxin (Fig. 25 D) avoided any fungal growth. Interestingly, the parental strain S238N and the monokaryotic strain H70 were much more sensitive to hygromycin than the other strains. In contrast, increasing BASTA concentrations (Fig. 25 B) inhibited the growth of all tested *Laccaria* strains but did not abolished it completely. However, growth on BASTA resulted in a sectoral growth of all fungal strains.

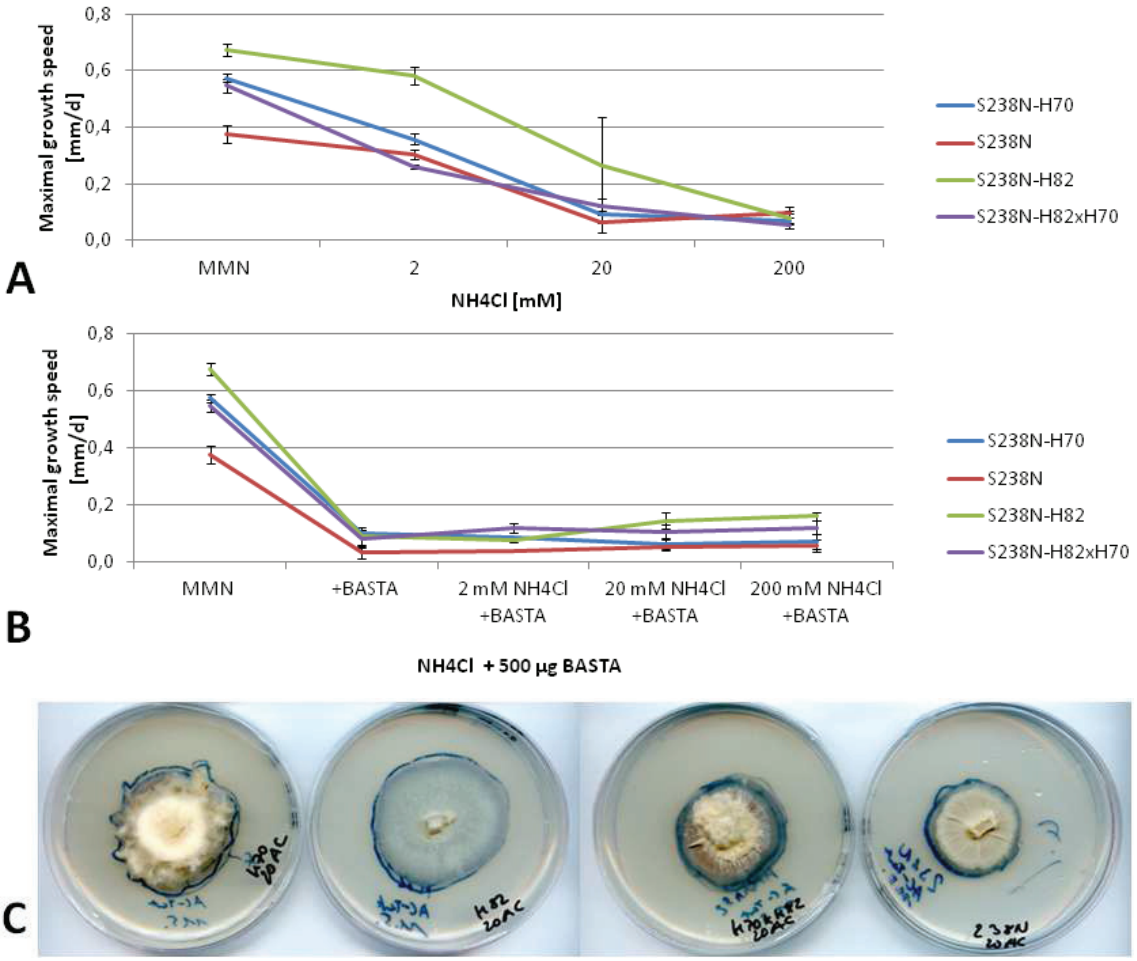


Fig. 26. Effects of BASTA and ammonium chloride on fungal growth behaviour
Growth speed (y-axis) of two mono- and two dikaryotic *Laccaria* strains on MMN medium supplemented with increasing NH_4Cl (A), or increasing NH_4Cl plus fixed BASTA (B) concentrations was investigated (x-axis). To illustrate strain-dependent growth differences photos of typical S238N-H70, S238N-H82, S238N-H82xH70, and S238N colonies on agar plates containing 20 mM NH_4Cl without BASTA are displayed (C).

BASTA-dependent growth inhibition is based upon intracellular ammonium toxicity (Hoerlein 1994). Therefore, exogenous elevated ammonium together with a fixed (500 µg/mL) BASTA concentration in the growth medium was investigated. The

addition of NH_4Cl, however, did not increase BASTA-dependent growth inhibition (Fig. 26 B), but its application (20 mM) without BASTA caused strong retardations in growth speeds (Fig. 26 A). Especially for H70 (Fig. 26 C, first plate on the left) a strong phenotypical change was observed, as prior also discovered during the $(NH_4)_2HPO_4$ treatment, characterised by an intense aerial colony.

3.4 Construction of fungal transformation vectors

3.4.1 Isolation of genomic DNA from *Laccaria bicolor*

*Laccaria bicolo*r genomic DNA, serving as a template for PCR amplification of selected genes, was isolated from S238N mycelium grown in a MMN-based liquid culture using CTAB protocol (see 2.5.1.2). A typical gDNA sample is shown in Fig. 27.

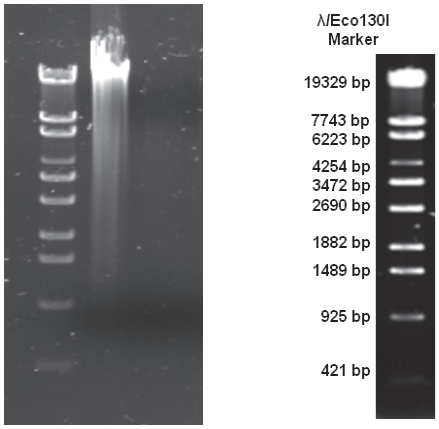


Fig. 27. Isolated genomic DNA of *L. bicolor*
Genomic DNA was isolated from *Laccaria* mycelium grown in liquid culture. A 2 µL aliquot was separated using agarose gel-electrophoresis (0.8 % (w/v)) and stained with ethidium bromide; *Eco130I*-digested lambda (λ) DNA was used as a size marker.

3.4.1.1 Genomic DNA fragments as template for construction of inverted repeats

The four genes chosen for RNAi-based gene silencing were PCR-amplified in two approximately 2.6 kb large genomic DNA fragments, covering together the entire coding region and about 1000 bp up- and downstream of the 5'- and 3'-ends (see Fig. 28 and chapter 2.5.4.6). This strategy was chosen because amplification of larger fragments drastically decreased PCR efficiency.

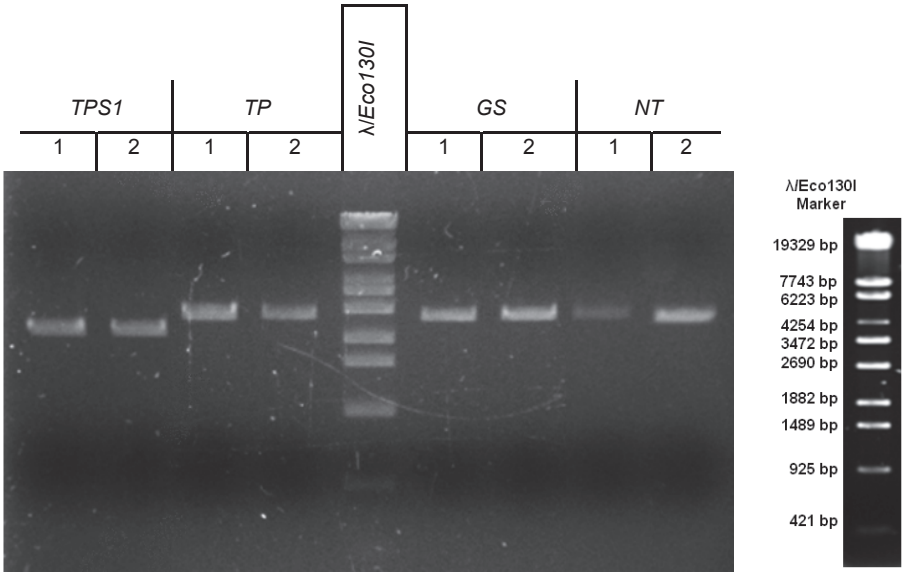


Fig. 28. PCR-amplified genomic DNA fragments of selected genes
Aliquots of gel purified DNA fragments, obtained from PCR reactions using a proof-reading Taq polymerase (see 2.5.2) and gene-specific primer pairs (Tab. 5), were subjected to analytical gel electrophoresis. The first two lanes display the *TPS1* fragments, the third and forth lane contain both *TP* fragments, in lanes six and seven the *GS* fragments, and in lanes eight and nine the *NT* amplicons are exhibited. *Eco130I*-digested lambda DNA was used as a size marker.

The purified DNA fragments were integrated into the pJet1.2/blunt vector. Analytical restriction analysis was performed to proof the orientation of the integrated DNA fragments (Fig. 29).

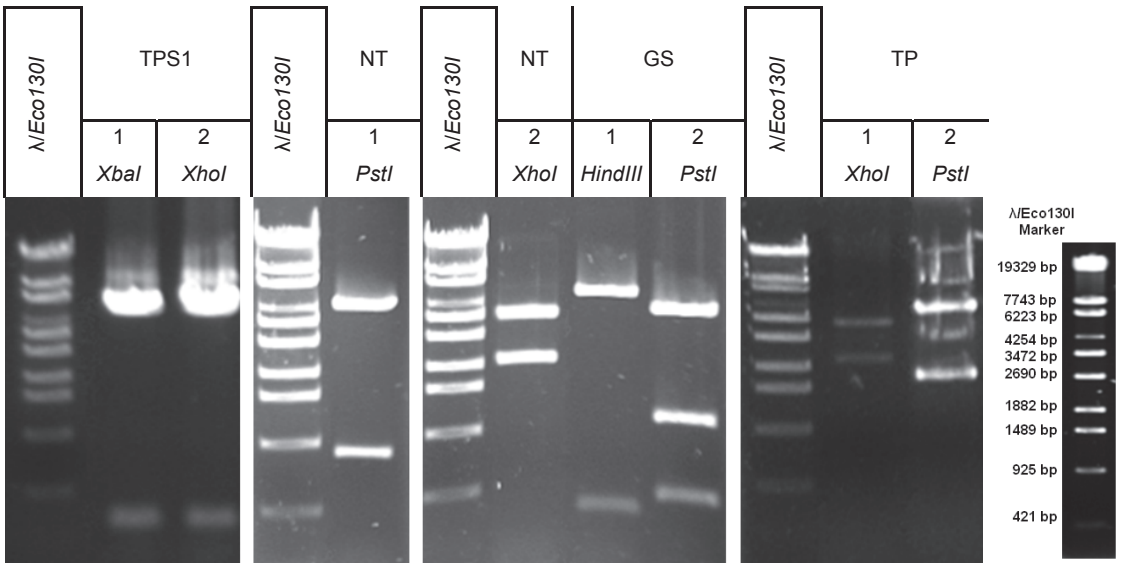


Fig. 29. Orientation of selected PCR-amplified genes in the pJet1.2 entry vector
Displayed are electrophoretically separated analytic restrictions of pJet1.2 plasmids with integrated *Laccaria* gDNA fragments. For each indicated enzyme (TPS1, NT, GS, and TP) both amplified and ligated fragments are displayed (1, 2). The enzymes for the digestions are also mentioned in the description of each lane. As DNA ladder *λ/Eco130I* was introduced for DNA

electrophoresis. First two digested pJet1.2 plasmids TPS1-1 (*XbaI*-digested, lane two) and TPS1-2 (*XhoI*-digested, lane three) demonstrate both amplified sense integrated TPS1 fragments, visible as each two fragments of 155 bp plus 4819 bp and 165 bp plus 4807 bp, respectively. In second gel, the *PstI*-hydrolysed plasmid NT-1 is shown with antisense oriented first *NT* fragment (fragments of 728 bp and 4751 bp). Second *NT* fragment containing plasmid (NT-2 in sense orientation) is displayed in second lane of gel three, *XhoI*-hydrolysed into 1913 bp and 3443 bp. Both *GS*-specific plasmids are visible in the next two lanes as antisense orientated GS-1 insert (digested with *HindIII* into 313 bp and 5177 bp) and antisense GS-2 (*PstI*-hydrolysed into fragments of 407 bp, 1092 bp, and 3979 bp). In lane two of the last displayed gel on the right site the antisense TP-1 plasmid is visible, digested by *XhoI* into 2153 bp and 3313 bp. Last lane demonstrates the sense TP-2 carrying plasmid, digested through *PstI* resulting in 1566 bp and 3926 bp fragments.

The most efficient RNAi-based gene silencing strategy is to generate short (few hundred bp long) inverted repeats separated by a spliceable intron (Smith, Singh et al. 2000; Goldoni, Azzalin et al. 2004; Kemppainen, Duplessis et al. 2009). Thus, all JGI-predicted gDNA sequences of the four selected genes were analysed for most promising exon-intron combinations and for *TPS1* and *GS* the first exon region was chosen, whereas the second exon region was used in case of NT- and TP-specific RNAi constructs. Two PCR fragments were generated per gene; first fragment always contained a part of the protein coding region plus a proximate spliceable intron, while the second DNA fragment did only contain the coding region.

For generation of RNAi constructs, restriction enzyme digestion sites had to be introduced. The respective sites were included within PCR primers (see 2.5.4.7). In Fig. 30 the corresponding purified PCR amplicons of each gene are shown.

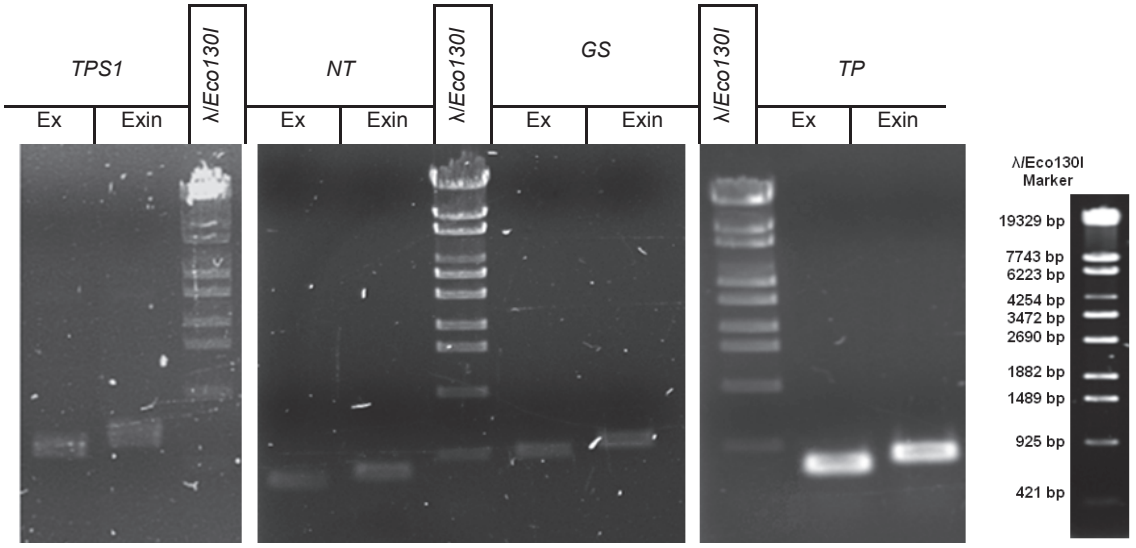


Fig. 30. PCR fragment for synthesis of RNAi cassettes
The DNA fragments were amplified using Phusion® Taq polymerase (see 2.5.2) and primers shown in Tab. 6. gDNA fragments are indicated by the corresponding abbreviations (*TPS1*, *NT*, *GS,* and *TP*). In the left lane of each block always the shorter fragment (that does not contain the intron sequence; Ex) and in the right lane each the corresponding prolonged Exin was

loaded. The expected fragment sizes are listed in Tab. 6. Marker λ/*Eco130I* served as DNA ladder for DNA electrophoresis.

After successful amplification, the purified DNA fragments were cloned into pJet1.2. Inverted repeats of the genes of interest, separated by the selected intron, were generated in the pJet1.2 vector backbone (described in Fig. 31) and transformed into the *E. coli* SURE strain to avoid rearrangement.

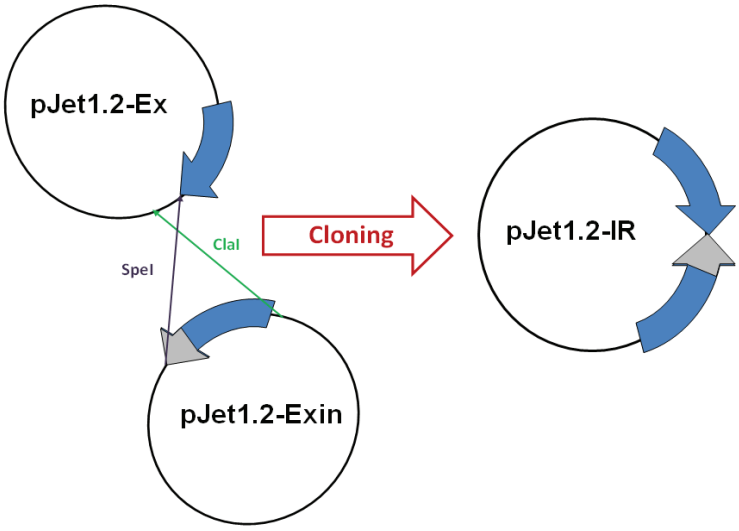


Fig. 31. Construction scheme to generate inverted repeats
The plasmids pJet1.2-Ex and pJet1.2-Exin are containing a given gene fragment without (Ex) or with intron (Exin) in defined orientations. The exon sequence is marked by a light blue arrow, the intron by a grey arrowhead. Both constructs were digested with the restriction enzymes *SpeI* and *ClaI*. In case of pJet1.2-Ex a small vector fragment of approximately 100 bp was released, while in case of pJet1.2-Exin the insert was liberated. After gel purification the Exin insert was integrated into the linearised pJet1.2-Ex, resulting in two repeats of the coding region, now oriented as inverted repeat, separated by the intron in a pJet1.2 backbone (pJet1.2-IR).

This strategy could be followed for all constructs except that of TP. Here another strategy was necessary, because of an internal *ClaI* recognition site within the intron sequence (for details see Fig. 32).

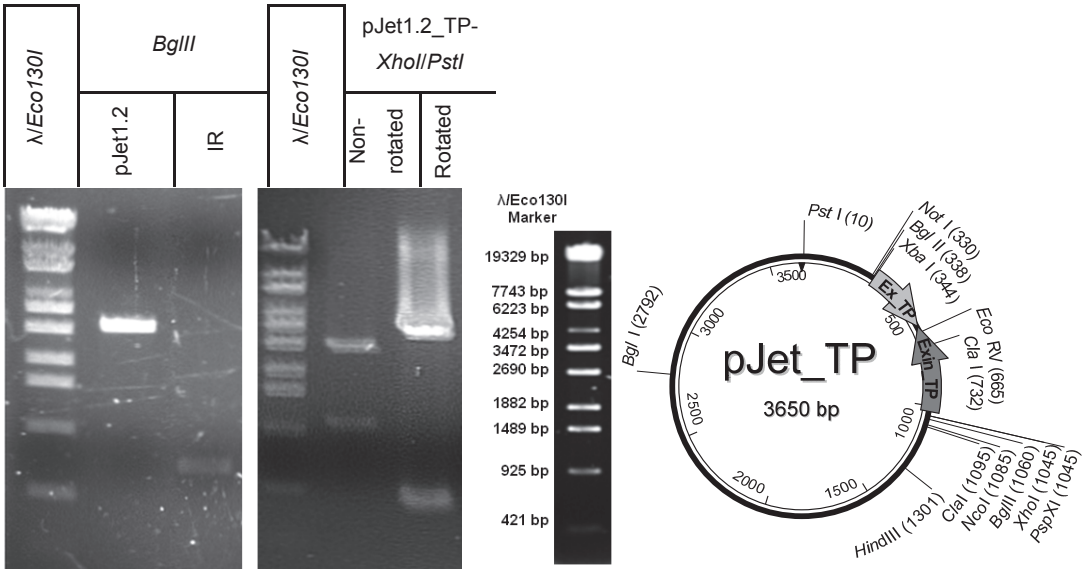


Fig. 32. *TP*-specific inverted repeat creation within the pJet1.2 plasmid
A primer-located *SpeI* recognition site was introduced into the amplified DNA fragments. *SpeI* generates overhangs that are compatible to an *XbaI* recognition site, present in the pJet1.2 backbone. The TP-Exin fragment containing plasmid was linearised by *XbaI*/*NcoI* double digestion and a purified *SpeI*/*NcoI* fragment of TP-Ex was integrated. Unfortunately, this strategy leads to a wrong orientation of the IR. To change its orientation, the entire inverted repeat (722 bp) was released by two pJet1.2-located *BglII* restriction sites (third lane of first electrophoresis), flanking the MCS of the vector, and religated into the dephosphorylated pJet1.2 backbone (visible in first gel in lane two as 2928 bp fragment). The two possible pJet-TP variants with different orientations are displayed in second gel (digested with *XhoI* and *PstI*). First visible plasmid contains the false oriented fragment, regarding the two fragments of 1035 bp and 2615 bp, while the second plasmid comprehends the aspired oriented IR resulting in fragments of 343 bp and 3307 bp. *Eco130I*-digested lambda DNA was used as a size marker. A schematic view of the aspired plasmid is pictured on the right with indicated applied restriction enzyme recognition sites.

The final constructs were analysed by restriction digestions as well as sequencing to check for their accuracy of IR construction.

3.4.2 Integration of inverted repeats into a fungal transformation vector

For strong and constitutive expression in fungi, the inverted repeats were supposed for cloning between the *glyceraldehydes-3-phosphate dehydrogenase* (*gpd*) promotor of *Agaricus bisporus* and a 35S-transcription terminator sequence. Unfortunately, this could not directly be performed within the final destination vector pBGgHg due to additional restriction enzyme recognition sites within the vector backbone. Therefore, first the eGFP expression cassette of pBGgHg was excised by *SacI*/*SalI* double digestion and cloned into the pBluescript II SK (+) plasmid, which was previously linearised by the same enzyme combination, resulting in a pBluescript variant with

integrated eGFP expression cassette. Then, eGFP coding sequence was removed by double digestion with *NotI*/*NcoI* and the different inverted repeats (previously excised by the same enzyme combination) were integrated giving rise to NT-, GS-, TP-, and TPS1-RNAi expression cassettes in a pBluescript backbone (Fig. 33).

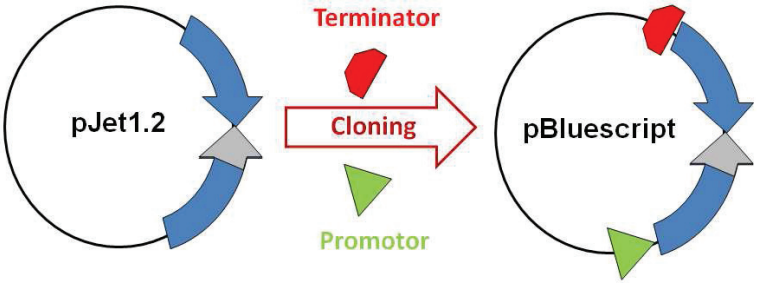


Fig. 33. Generation of RNAi expression cassettes in pBluescript
The eGFP expression cassette of pBGgHg was introduced into pBluescript II SK (+) (pBluescript) by *SacI*/*SalI* double digestion. The reading frame of eGFP was removed by double digestion with *NotI*/*NcoI* and the inverted repeats (grey arrow head indicates intron, blue bars exons) released from pJet1.2 by double digestion with *NotI*/*NcoI* were inserted into pBluescript.

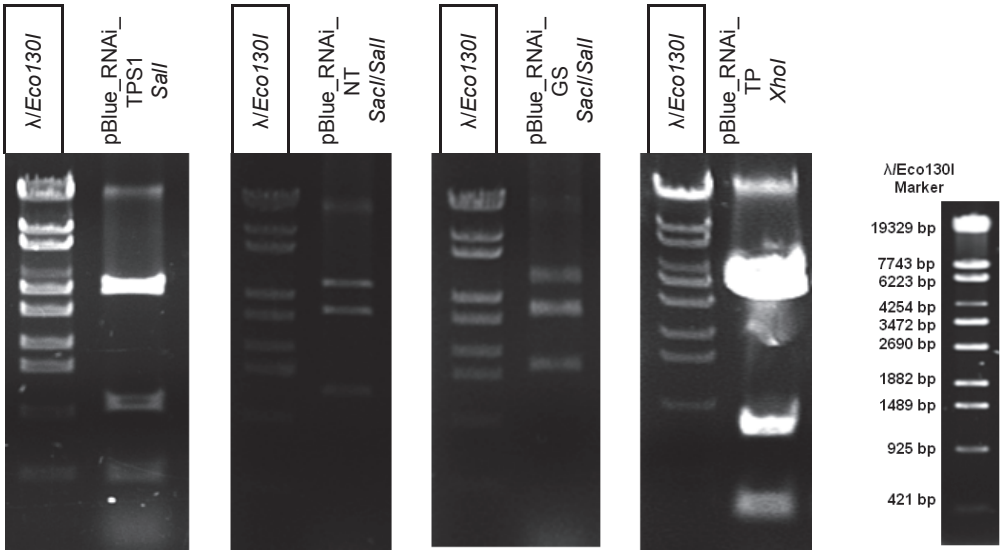


Fig. 34. Proof of IR ligation between *gpd* promotor and 35S-terminator
The produced pBluescript-based plasmids were tested via restriction analysis and electrophoresis, as demonstrated above. The first plasmid, in first gel, is a *SalI*-digested pBluescript (pBlue_RNAi_TPS1) containing the RNAi expression cassette for TPS1 (fragments of 355bp, 923 bp, and 3309 bp). Second plasmid (pBlue_RNAi_NT), visible in second gel, displays a *SacI*/*SalI* digestion of an NT-RNAi cassette carrying pBluescript (1191 bp RNAi cassette plus backbone of 2876 bp). The third visible fragment (size close to the 4254 bp marker fragment) indicates a partial restriction as linearised plasmid of 4068 bp. In third gel the GS-specific RNAi pBluescript (pBlue_RNAi_GS) is demonstrated, hydrolysed by *SacI*/*SalI* (1555 bp cassette plus backbone of 2876 bp), and again a residual linearised plasmid. pBlue_RNAi_TP indicates a TP-specific cassette carrying pBluescript, digested with *XhoI* and further analysed in fourth gel (207 bp, 744 bp, and 3219 bp). Marker λ/*Eco130I* was introduced as DNA ladder for DNA electrophoresis.

Analytical restriction analysis was carried out to proof correct cloning (Fig. 34).

3.4.3 Construction of binary vectors for fungal transformation

Because rearrangement of inverted repeats is frequently observed in conventional *E. coli* strains, the SURE strain was exclusively used for further IR cloning purposes. As the SURE strain harbours genomic encoded kanamycin resistance, an additional dominant selection marker for *E. coli* transformation (spectinomycin resistance) had to be integrated into the *Agrobacterium*-based fungal transformation vector pBGgHg (Fig. 35).

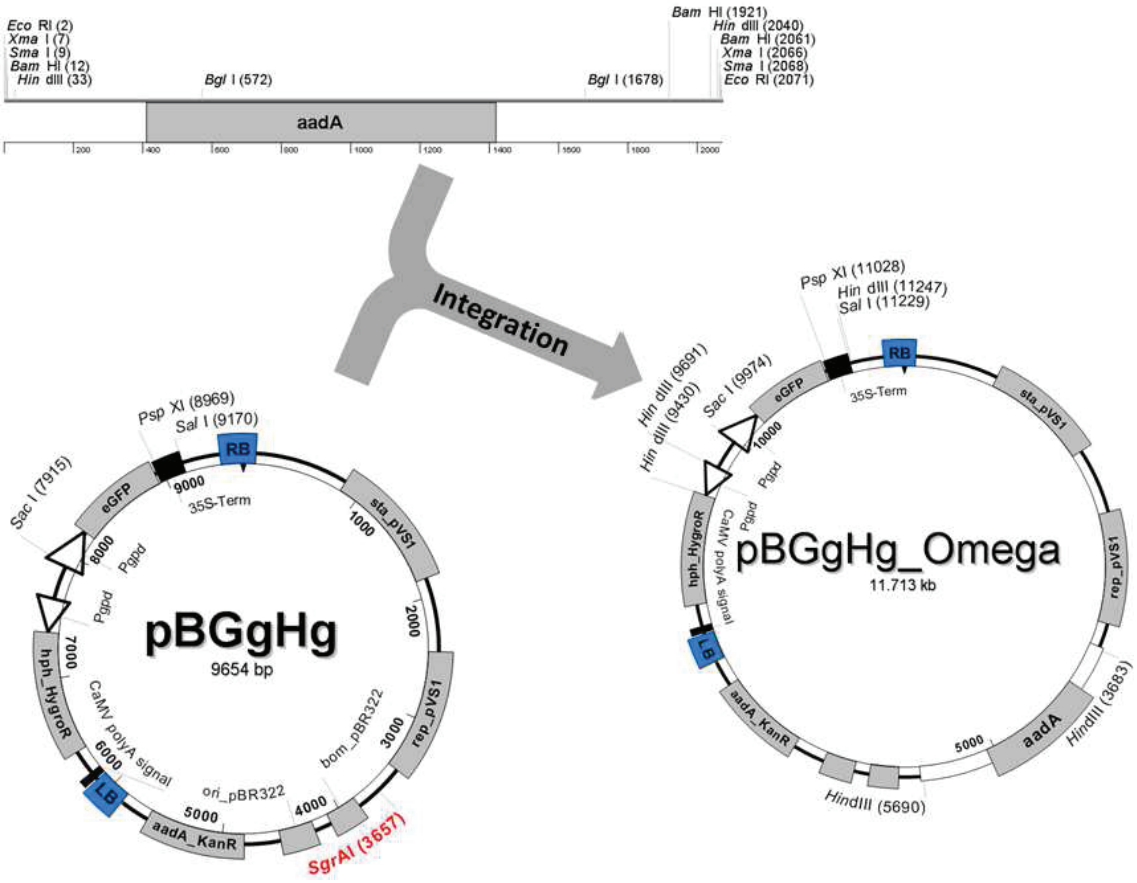


Fig. 35. Integration of a spectinomycin resistance cassette into the pBGgHg vector backbone
The backbone of the binary vector pBGgHg was linearised by *SgrAI* digestion (red highlighted) without disruption of any functional sequences. pBGgHg-specific functional elements can be followed regarding Fig.12 on page 36 (pBGgHg vector map). The spectinomycin resistance cassette (aadA) was excised from the plasmid pHP45Ω using *XmaI* (generates *SgrAI* compatible overhangs) and integrated into pBGgHg resulting in pBGgHg_Omega.

For modification a unique pBGgHg backbone-located *SgrAI* digestion site (at position 3657; red indicated in Fig. 35) was chosen, because of its induced overhangs allowing

the ligation of *XmaI*-hydrolysed fragments. Accordingly, *XmaI* digestion of plasmid pHP45Ω (Prentki and Krisch 1984) was conducted, resulting in the excision of the spectinomycin resistance cassette (Ω-fragment) which was purified. Then, pBGgHg was hydrolysed by *SgrAI* and dephosphorylated to avoid recirculation. The *XmaI* excised Ω-fragment of pHP45Ω was integrated into pBGgHg, giving rise to pBGgHg_Omega (pBGΩ). Analytical restriction analysis was performed to proof the construct and to determine the orientation of pBGΩ (Fig. 36).

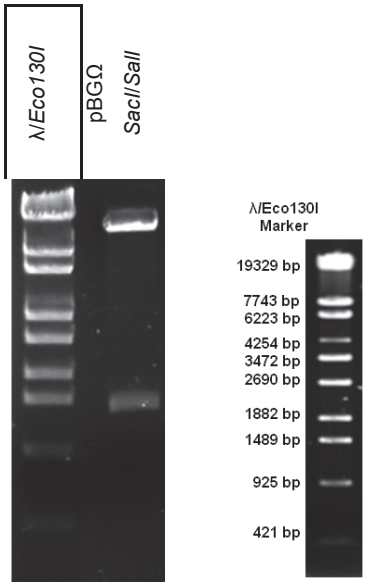


Fig. 36. Construction and verification of pBGgHg_Omega
The generated pBGgHg derivate with integrated Ω-fragment (of pHP45Ω) is displayed in the figured electrophoresis, double-digested by *SacI* and *SalI*. This restriction divided the plasmid into a 1255 bp (full eGFP cassette) fragment and a residual 10458 bp fragment. *Eco130I*-digested λ DNA was used as a size marker.

For the integration of the RNAi cassettes into pBGgHg_Omega, the eGFP expression cassette was removed by *SacI*/*SalI* double digestion. Then the NT-, GS-, and TP-RNAi expression cassettes were released from the respective plasmids also by *SacI*/*SalI* double digestion. The cassettes were isolated by gel electrophoresis and gel extraction and were further ligated into the linearised pBGΩ vector (Fig. 36) giving rise to the respective fungal transformation vectors (as demonstrated in Fig. 38 and Fig. 39).
Unfortunately, the integration of the TPS1-RNAi cassette into pBGΩ was not applicable by this strategy due to a *SalI* recognition site within the cassette. Therefore, pBGgHg_Omega was linearised with *SacI* and *PspXI*, leaving the 35S-terminator of the eGFP cassette within the vector. Then, the TPS1-RNAi fragment was excised with the same enzyme combination, purified by gel electrophoresis and gel extraction, and

ligated into the linearised pBGgHg_Omega vector. Fig. 37 shows the construction of TPS1-RNAi-specific pBGΩ and its proof.

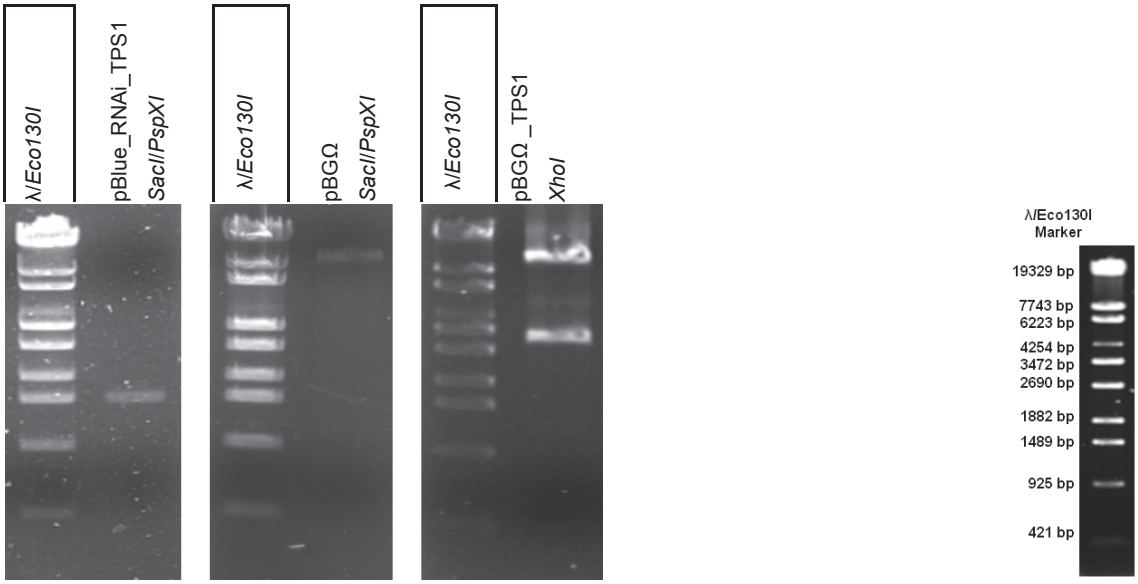


Fig. 37. Construction and proof of TPS1 RNAi pBGgHg_Omega
The first gel shows the purified TPS1-RNAi cassette (1458 bp), while second gel displays the purified pBGΩ backbone (10659 bp). Analytic *XhoI* digestion was performed to proof the final TPS1 RNAi pBGgHg_Omega construct. Two DNA fragments 3099 bp and 9062 bp in size were expected (third gel). As DNA ladder, the λ/*Eco130I* was introduced for DNA electrophoresis.

Plasmid maps of all four final constructs are shown in Fig. 38 and analytic restriction analysis to proof proper construction is shown in Fig. 39.

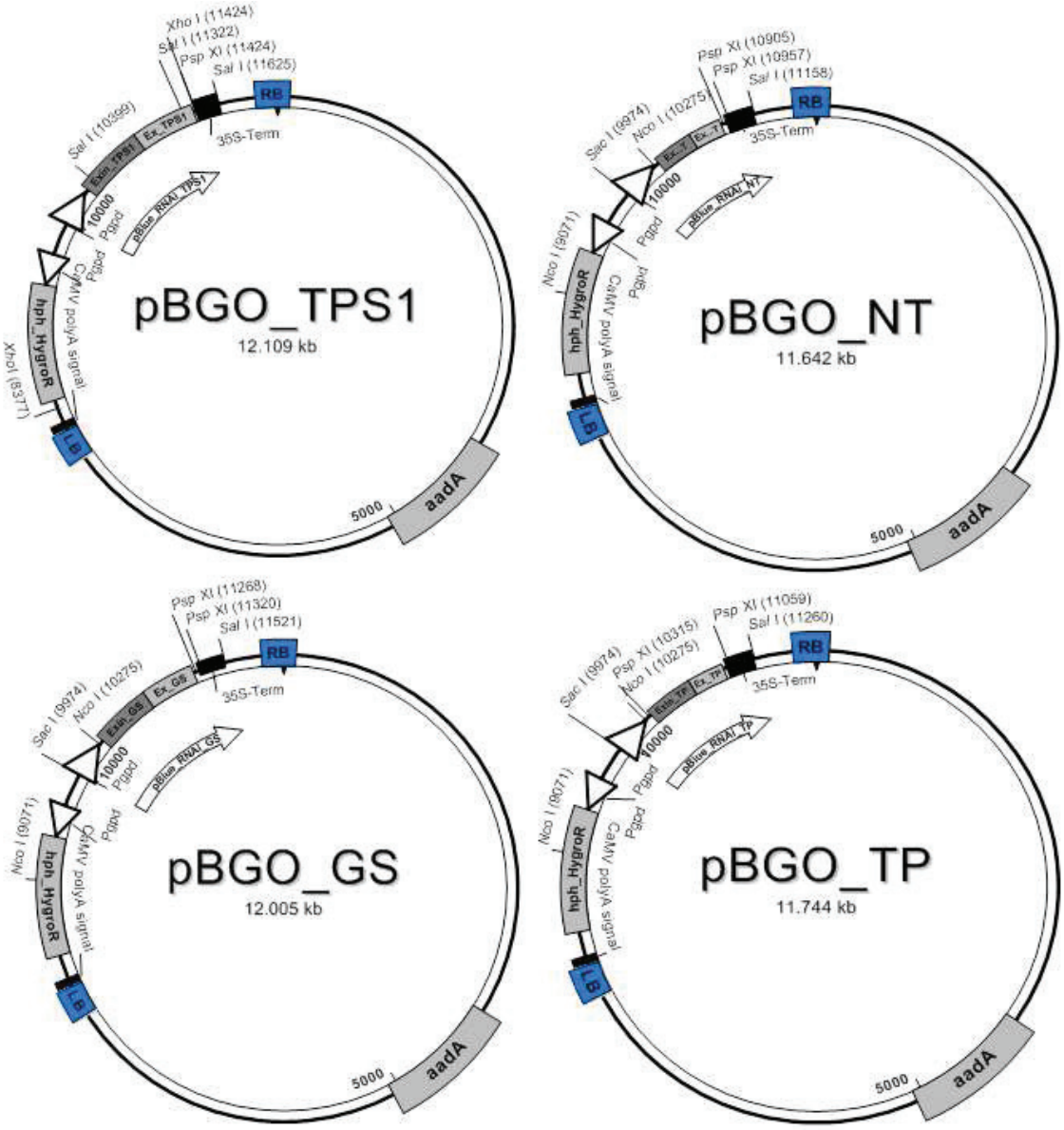


Fig. 38. Binary vectors containing gene-specific RNAi cassettes for *Laccaria* transformation

Schematised are vector maps of fungal transformation plasmids for gene silencing attempts (pBGO = pBGΩ) with functional important elements, as left and right t-DNA borders (LB, RB), gene-specific inverted repeats (Exin-Ex structures), promotor regions (white triangles), terminators (black boxes), the hygromycin resistance cassette (hph_HygroR), and the newly introduced spectinomycin resistance cassette (aadA). Furthermore, restriction endonucleases sites necessary for cloning purpose together are also highlighted.

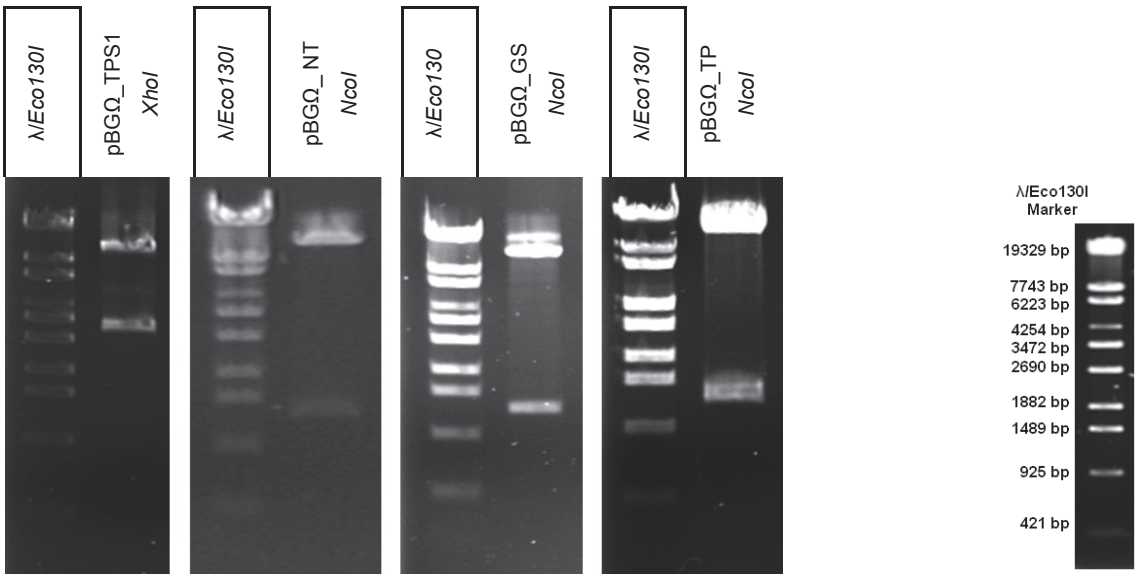


Fig. 39. Analytic restriction enzyme digestion to proof proper construction of fungal RNAi transformation vectors
RNAi pBGgHg_Omega constructs were applied to analytic restrictions analysis. First gel displays the *XhoI*-digested TPS1-specific pBGgHg_Omega (pBGΩ_TPS1) giving rise to two DNA fragments 3047 bp and 9062 bp in size. pBGΩ_NT was hydrolysed via *NcoI* to obtain the expected fragments of 1204 bp and 10438 bp (second gel). In the third gel, the pBGΩ_GS variant was separated, digested again with *NcoI* to obtain the same 1204 bp fragment plus a 10801 bp fragment. In last displayed gel the plasmid pBGΩ_TP was analysed, also *NcoI*-treated to reveal the demanded fragments 1204 bp plus and 10540 bp.

3.5 Transformation of *Agrobacterium tumefaciens*

All RNAi pBGΩ constructs were transformed into *Agrobacterium tumefaciens* (strains AGL1, LBA4404, and C58/PMP90). Chemical as well as electro competent bacteria were used for transformation; kanamycin resistance was used as dominant selection system. To proof the presence of binary vectors, a 402 bp fragment of the hygromycin resistance gene was PCR-amplified (Fig. 40) from progenies of single kanamycin resistant colonies, using Hygro-for and -rev (appendix Tab. 19) primers.

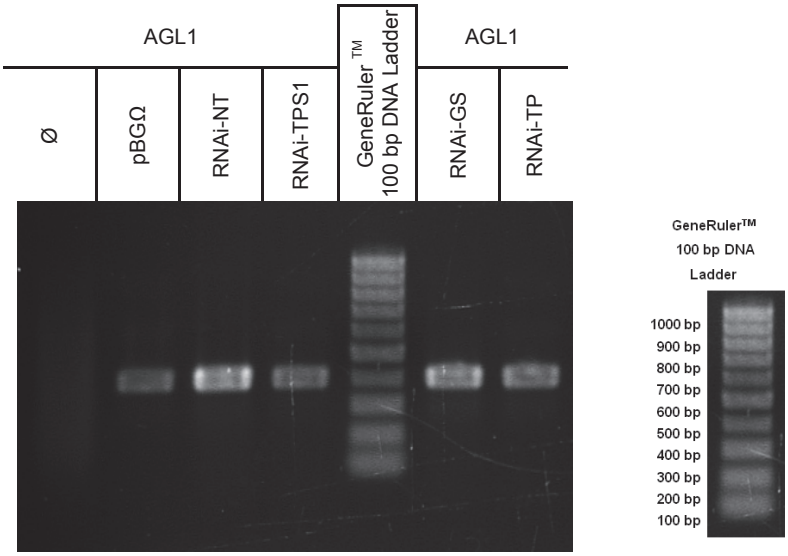


Fig. 40. PCR-amplified gene fragments of hygromycin resistance cassettes of transgenic *A. tumefaciens* (AGL1)
To verify bacterial transformation, single transformed agrobacterial colonies were resuspended in 100 µL water. 0.5 µL cell suspension was applied to 20 µL PCR reactions using Hygro -for and -rev primers. Aliquots of the PCR reaction were applied to gel electrophoresis and ethidium bromide staining. In the first lane non-transformed AGL1 cells were used for PCR while in lanes two to four and six to eight bacteria containing pBGΩ, pBGΩ_NT, pBGΩ_TPS1, pBGΩ_GS, and pBGΩ_TP were used as template. In lane five GeneRuler^{TM} 100 bp DNA Ladder was loaded.

3.6 Transformation of *Laccaria bicolor*

Approximately 25 independent transformants of the two monokaryotic *Laccaria bicolor* strains S238N-H82 and S238N-H70 were obtained for any of the four RNAi (TPS1, NT, TP, or GS) constructs and control transformants containing the empty pBGgHg_Omega vector, leading to a total of 270 independent hygromycin resistant fungal transformants.

Starting from the transformation protocol for *Agaricus* (Chen, Stone et al. 2000) and for *Laccaria,* published by Kemppainen (Kemppainen, Circosta et al. 2005) that did not give rise to any fungal transformant, several protocol improvements were tested. These modifications originate from publications about agrobacterial transformation of *Saccharomyces cerevisiae, Agaricus bisporus,* and other filamentous fungi (Bundock, den Dulk-Ras et al. 1995; Bundock and Hooykaas 1996; van de Rhee, Graca et al. 1996; de Groot, Bundock et al. 1998; Gouka, Gerk et al. 1999; Chen, Stone et al. 2000; Hanif, Pardo et al. 2002; Combier, Melayah et al. 2003; Burns, Gregory et al. 2005; Burns, Leach et al. 2006; Samils, Elfstrand et al. 2006) as well as plant transformation protocols (Lazo, Stein et al. 1991; Clough and Bent 1998; Bent 2000; Hwang and Gelvin 2004; Jones, Doherty et al. 2005; Gelvin 2006). The tested conditions and

media to aim for t-DNA transfer into monokaryotic *Laccaria bicolor* strains are listed in Tab. 20 and 21 of the appendix section. The transformation procedure can be divided into three main parts, pre-cultivation of bacteria and fungi, the transformation process, and selection of hygromycin resistant fungal hyphae. Only the final transformation protocol was listed in the material and methods section.

First successful transformation attempts revealed only a low transformation efficiency of about 1.4 % in August 2010. This efficiency resulted from the ratio of three transformed colonies per overall 210 introduced colonies (converted into percent). Furthermore, these three transformants are 1.11 % of all achieved transformants during this PhD project (270 individual transformants). Here, one problem was a stimulated hygromycin resistance of non-transformed *Laccaria* hyphae over time. Consequently, the hygromycin concentration was nearly doubled to ensure sufficient selection pressure over time. The final transformation protocol leaded to transformation efficiencies of up to 16.4 % (131 transformants of 800 treated fungal colonies in June 2011). This high number of transformants is reflected in the high amount of the overall achieved transformants at this date (almost 48 % of all 270 generated strains). Consequently, these conditions and compositions of utilised media are most potent to trigger t-DNA transfer into the fungal hyphae. In Fig. 41 a selective MMN plate after a successful transformation attempt is displayed; the young hygromycin resistant colonies are nicely visible as small white colonies.

Fig. 41. Selective petri dish with young hygromycin resistant *Laccaria* colonies
Displayed is a photographed selective agar plate after 8 weeks of hygromycin selection. Small hygromycin resistant white fungal colonies were growing, while untransformed fungal hypha started to lyse (brownish agar plugs).

3.6.1 Phenotypical characterisation of transgenic *L. bicolor* strains

Always ten (out of a nearly 25) *Laccaria* transformants containing TPS1-, NT-, or TP-RNAi constructs were randomly selected and grown on MMN petri dishes supplemented with 25 mM glucose. The plates were covered by a cellophane membrane to allow mycelial harvest without agar contamination. The growing mycelial front was followed over a period of 21 days (labelled with a permanent-maker at given time points). For mycelial harvest fungal colonies were divided into three parts by scalpel-excision (Fig. 42), the outermost and youngest part (hyphal age up to 7 days old), the middle part (hyphal age 8 to 14 days, and the oldest part (hyphal age 15 to 21 days). Only the initial inoculum in the centre of the colony was excluded. Always eight biological replicates were performed for each transformant and 18 for the respective wild types. Mycelia of two agar plates were collected in 1.5 mL microreaction tubes, shock-frozen in liquid N_2, and lyophilised to determine the dry-weight of each sector and to be stored for following total RNA and metabolite extractions.

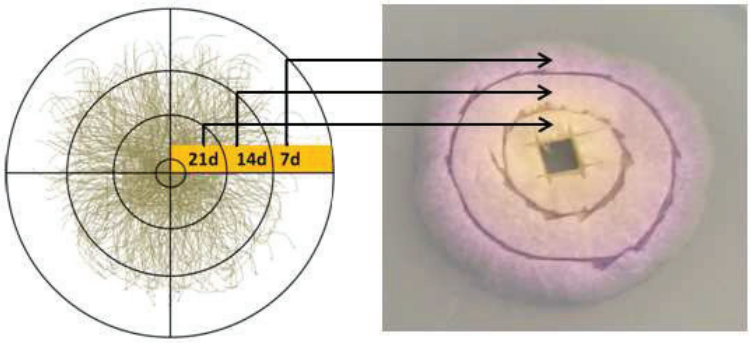


Fig. 42. *Laccaria bicolor* colony divided into three sectors of different ages
The fungal colony was divided into three circles, the outermost, youngest part (hyphal age up to 7 days), the middle part (hyphal age 8 to 14 days), and the oldest part (hyphal age 15 to 21 days).

3.6.2 Maximal growth speeds of fungi with modulated trehalose metabolism

Hyphal growth was followed over time by periodically marking the hyphal growth front. The increase in diameter of the fungal colony was determined for all four growth directions and used to calculate the maximal hyphal growth speed. The parental monokaryotic strains revealed different maximal growth rates between 1.006 mm/d (S238N-H82) and 1.141 mm/d (S238N-H70). Hence, maximal growth rate of transgenic

isolates was normalised to the corresponding wild type and is displayed as relative growth speed in percent of wild type growth.

In Fig. 43 the impact of TPS1-silencing is shown for ten independent transformants with S238N-H70 or S238N-H82 background. Eight out of ten TPS1-RNAi transformants with S238N-H70 background displayed a significantly reduced maximal growth speed (between 10 and 40 % reduction). In contrast, all transformants with S238N-H82 background showed a (not significant) tendency for increased maximal growth speed.

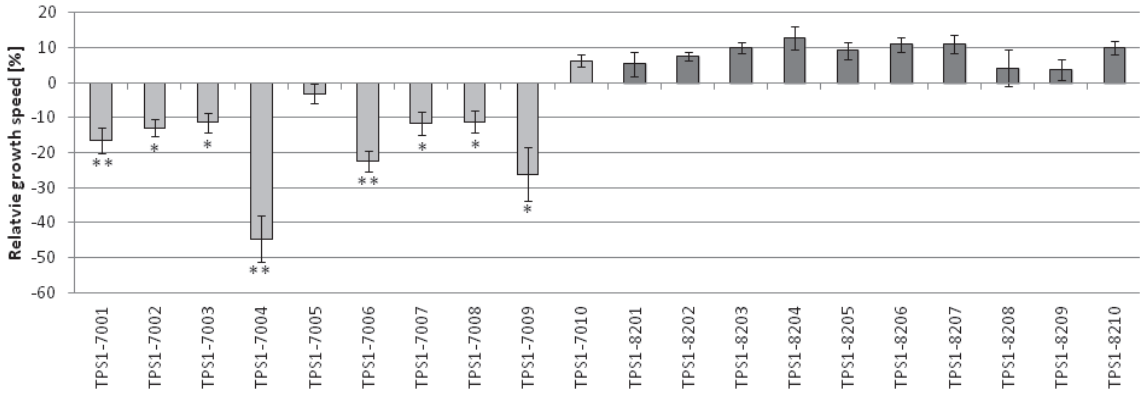


Fig. 43. Relative growth of independent TPS1-RNAi transformants in relation to the respective wild type

The relative growth speed (y-axis) for ten independent transformants per monokaryon (x-axis) was determined. Light grey bars indicate transformants with S238N-H70 background, while the dark grey bars show S238N-H82-transgenics. Calculated p-values are shown as stars (significant, p-value < 0.05 (*); highly significant, p-value < 0.01 (**); extremely significant, p-value < 0.001 (***)).

In Fig. 44 the impact of NT-RNAi (neutral trehalase-specific) on maximal growth speed of fungal hyphae is demonstrated. Independent of the fungal background (S238N-H70 or S238N-H82), a reduced maximal growth speed of fungal hyphae was observed for all investigated isolates upon transformation with the NT-specific silencing construct. While the observed reduction in the maximal growth speed was significant for nearly all isolates with S238N-H70 background, this was the case for only two out of ten isolates with S238N-H80 background. Growth reductions between 10 and 50 % were observed.

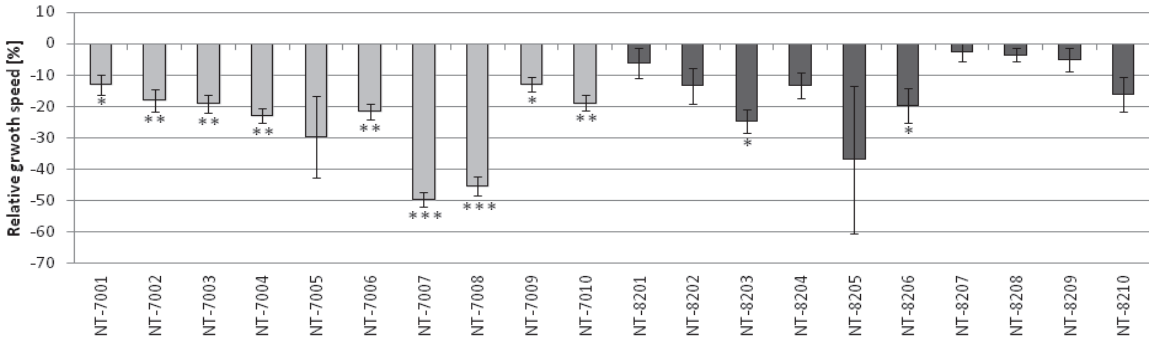


Fig. 44. Relative growth of independent NT-RNAi transformants in relation to the respective wild type
The relative growth speed (y-axis) of each 20 independent transformants (x-axis) compared to wild type hyphae was determined. Light grey bars indicate ten transformants with S238N-H70 background, while the dark grey bars show ten S238N-H82-transgenics. Calculated p-values are indicated as stars (significant, p-value < 0.05 (*); highly significant, p-value < 0.01 (**); extremely significant, p-value < 0.001 (***)).

In Fig. 45 differences in the maximal growth speeds of *Laccaria* monokaryons transformed with the TP (trehalose phosphorylase)-specific silencing cassette are shown.

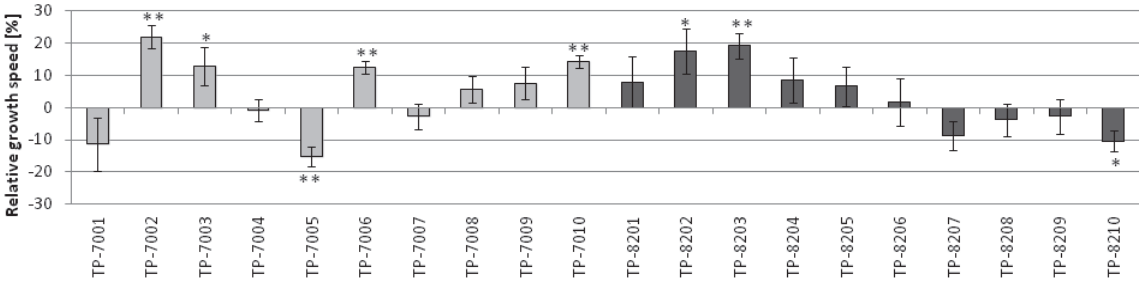


Fig. 45. Relative growth of independent TP-RNAi transformants in relation to the respective wild type
The relative growth speed (y-axis) for ten independent transformants (x-axis) per monokaryon was calculated. Light grey bars indicate transformants with S238N-H70 background, while the dark grey bars show S238N-H82-transgenics. Calculated p-values are shown as stars (significant, p-value < 0.05 (*); highly significant, p-value < 0.01 (**); extremely significant, p-value < 0.001 (***)).

In contrast to the two other constructs, transformation with the TP-RNAi cassette revealed no clear tendency. Transformants with S238N-H70 and S238N-H80 background showed both isolates with significant increased (up to 20 %) and decreased (up to 15 %) maximal growth speeds. Unfortunately, it was impossible to analyse GS (glycogen synthase)-specific transformants due to time constraints.

3.6.3 Hyphal densities of *Laccaria* strains

Next to the maximal growth speed, hyphal density is a criterion to phenotypically characterise fungal growth behaviour. For this task, the obtained growth area for each growth zone (1 to 3) was divided by the respective dry weight of fungal mycelia.
The densities (in mg/mm^2) of both wild type monokaryons are displayed in Fig. 46.

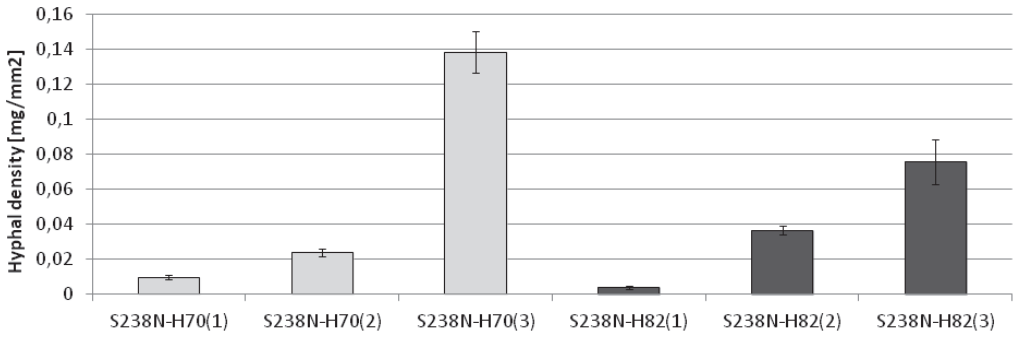


Fig. 46. Hyphal densities of the three areas (up to 21 days old mycelia) of *Laccaria* S238N-H70 and S238N-H82
On the y-axis, the obtained hyphal densities [mg/mm^2] are displayed. On the x-axis, the outermost area (7 days week old hyphae) is indicated as (1), the middle hyphal zone (8 to 14 days old hyphae) as (2), and the central part of the colony (up to 21 days old mycelium) as (3). Light grey bars indicated S238N-H70 hyphae and dark grey bars hyphae of S238N-H80.

While the hyphal density of S238N-H80 increased in an approximately linear manner over time, the oldest hyphae of S238N-H70 had a much higher density (nearly 14 times higher that of the youngest hyphae, indicated as 1 in Fig. 46). This indicates intense formation of aerial hyphae by this monokaryotic strain in the oldest sector of the fungal colony. Similar to maximal growth speed, S238N-H70 also revealed higher hyphal density than mycelia of S238N-H80.
Similar to the maximal growth speed, TPS1-RNAi transformants with S238N-H70 background showed a significantly reduced hyphal density, while it was increased in transformants with S238N-H80 background (Fig. 47).

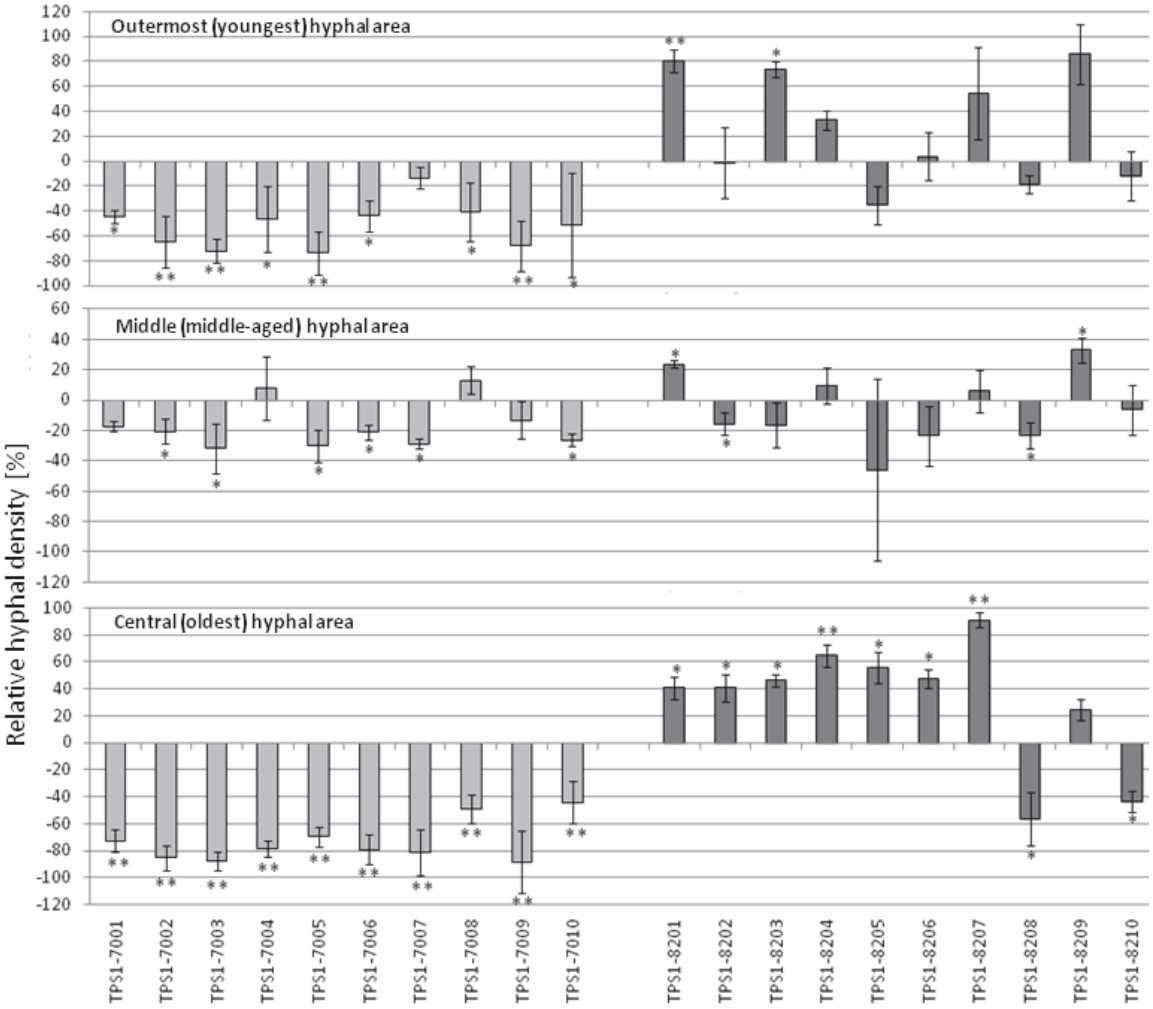


Fig. 47. Age-dependent changes in hyphal densities of *TPS1*-silenced *Laccaria* transformants
The relative hyphal density (y-axis) of ten independent transformants (x-axis) per monokaryon was determined. The three diagrams demonstrate the calculated differences in the outermost area (upper), middle area (middle), and central area (lower). Light grey bars indicate transformants with S238N-H70 background, while the dark grey bars highlight S238N-H82 transformants. Calculated p-values are displayed as stars (significant, p-value < 0.05 (*); highly significant, p-value < 0.01 (**); extremely significant, p-value < 0.001 (***)).

The smallest differences between transformants and their respective wild types were visible in the middle part of the fungal colony. Differences were most distinct in the oldest part of fungal colonies, where extensive formation of aerial hyphae was observed (most pronounced for hyphae with S238N-H70 background).

Similar to the maximal growth speed, all (oldest part of the colony) or nine out of ten (youngest part of the colony) TPS1-RNAi transformants with S238N-H70 background were significantly reduced in hyphal density. In contrast, seven (out of ten) transformants in the oldest and two (out of ten) in the youngest part of the colonies showed increased hyphal densities in transformants with S238N-H82 background.

Hyphal density and maximal fungal growth speed were in accordance for transformants expressing NT-RNAi silencing cassettes in the S238N-H70 background. As in the case of TPS1, the picture was more distinct in the oldest part of the fungal colony (Fig. 48). For transformants with S238N-H80 background, this is true only for mycelia of the middle and the central (oldest part) of the fungal colony in which five strains revealed significant reductions. In contrast, half of the isolates revealed (significantly) increased mycelial densities in the youngest part of the fungal colony.

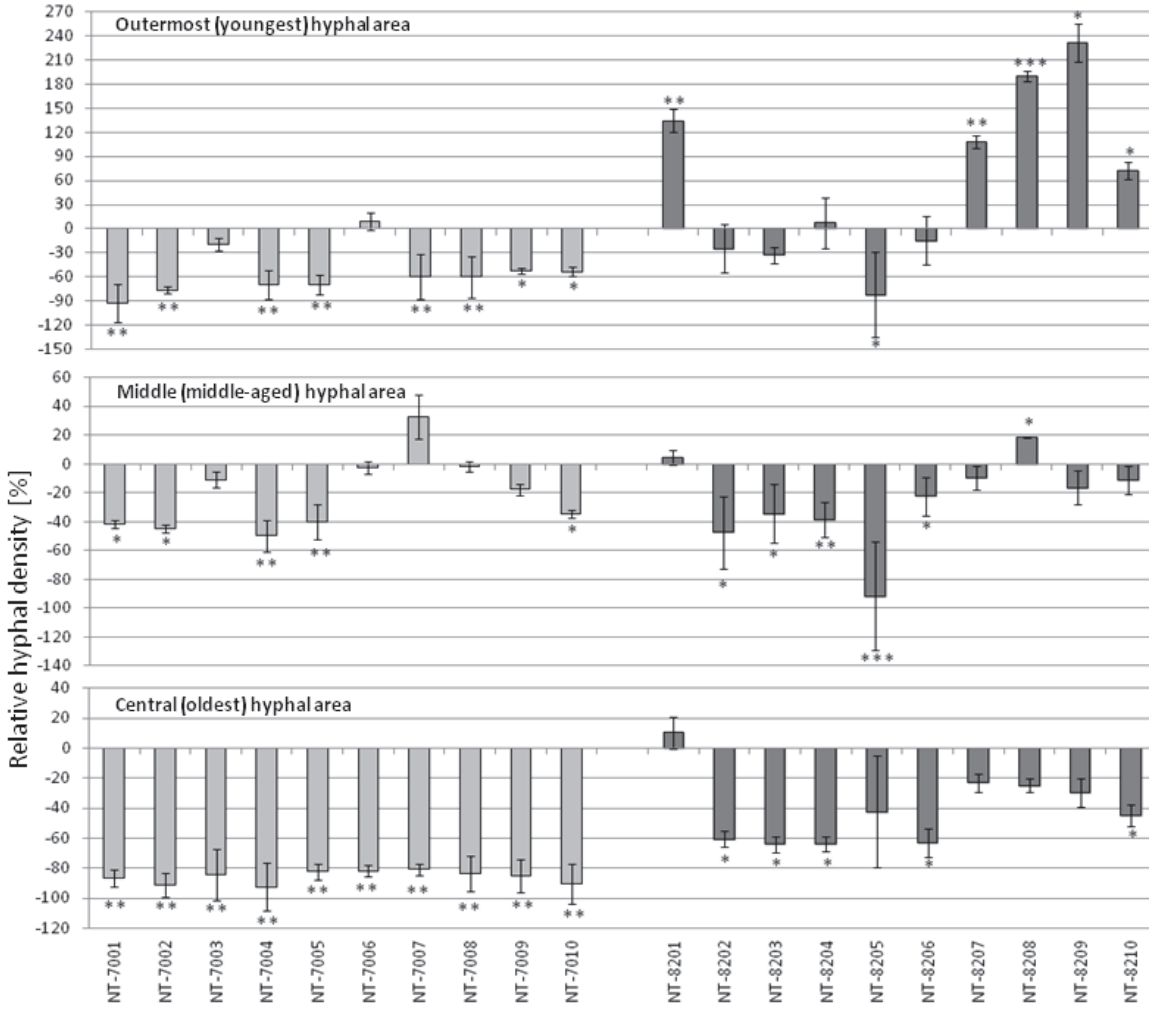


Fig. 48. Age-dependent changes in hyphal densities of *Laccaria* transformants containing NT-RNAi constructs
The relative hyphal density (y-axis) of independent transformants (x-axis) per monokaryon was determined. The three diagrams demonstrate the calculated differences in the outermost area (upper), middle area (middle), and central area (central). Light grey bars indicate transformants with S238N-H70 background, while the dark grey bars show S238N-H82-transgenics. Calculated p-values are shown as stars (significant, p-value < 0.05 (*); highly significant, p-value < 0.01 (**); extremely significant, p-value < 0.001 (***)).

3.6.4 Quantification of RNAi-based gene suppression in *Laccaria* transformants

3.6.4.1 Calibration curves and efficiency

Transcript levels of *TPS1*, *NT*, *TP*, *GS,* and *GP* were determined by quantitative RT-PCR (qPCR) using gene-specific primer pairs (Tab. 7) under optimised PCR conditions (Tab. 8). Putative contamination of first strand cDNA with genomic DNA could be visualised by generation of fragments with a different length. PCR fragments from genomic DNA would be 50 bp longer than those of cDNA. However, contamination with genomic DNA was never observed after rDNase content was increased by 20 % during total RNA preparation. Gene expression was calibrated to 18S rRNA content in the different RNA preparations.

For determining of gene expression by qPCR the amplification efficiencies of the gene of interest and of the reference have to be similar (Liu and Saint 2002; Wong and Medrano 2005). The efficiency of a given PCR reaction can be determined through standard curves and linear regressions (Pfaffl 2001; Nolan, Hands et al. 2006). For this purpose, purified PCR fragments were obtained from the chosen genes and DNA content was quantified photometrically. Dilutions ranging from 10^{-3} to 10^{-8} (up to 10^{-9} for 18S rRNA) were generated and applied to PCR. The obtained calibration curves are displayed in Fig. 49.

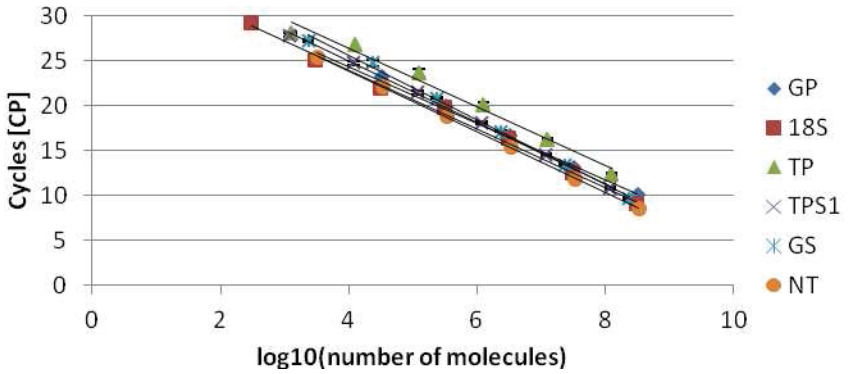


Fig. 49. Determination of PCR efficiencies for the different primer pairs used in this study
Dilution series (10^{-3} up to 10^{-8}; for 18S up to 10^{-9}) of purified PCR fragments with known DNA content were used for amplification in a LightCycler®480II. The obtained CP values (y-axis) were drawn against the molecule numbers (x-axis, logarithmic scale). Linear regression was used to calculate amplification efficiencies.

Calculated slopes of linear regressions can be directly used to evaluate the efficiencies (E) ($E = 10^{(-1/slope)}$) as listed in Tab. 13. Slopes between -3.2 and -3.5 indicates optimal

conditions resulting in nearly 100 % PCR efficiency (Meuer, Wittwer et al. 2001; Pfaffl 2001; Wittwer, Hahn et al. 2004; Nolan, Hands et al. 2006).

Tab. 13. Linear equations of regression lines and determination of PCR efficiency

Gene	Linear equation	R^2	Slope	Efficiency	Percent [%]
GP	-3,143x + 36,94	0,9956	-3,143	2,080	104,025
18S	-3,2608x + 37,036	0,9956	-3,2608	2,026	101,308
TP	-3,2563x + 39,459	0,9817	-3,2563	2,028	101,407
TPS1	-3,4181x + 38,64	0,999	-3,4181	1,961	98,069
GS	-3,591x + 39,943	0,9968	-3,591	1,899	94,939
NT	-3,3875x + 37,419	0,9999	-3,3875	1,973	98,667

PCR reactions were further analysed in 2 % (w/v) agarose gels (Fig. 50).

Taken together, all primer pairs allowed a high-specific fragment formation. With the exception of *TP*- and *GP*-specific dilution series, where high template concentration resulted in the formation of unspecific DNA fragments (visible as smear-like), no or only minor by-products were obtained. However, high template quantities necessary to form by-products in the dilution series were never obtained from cDNA.

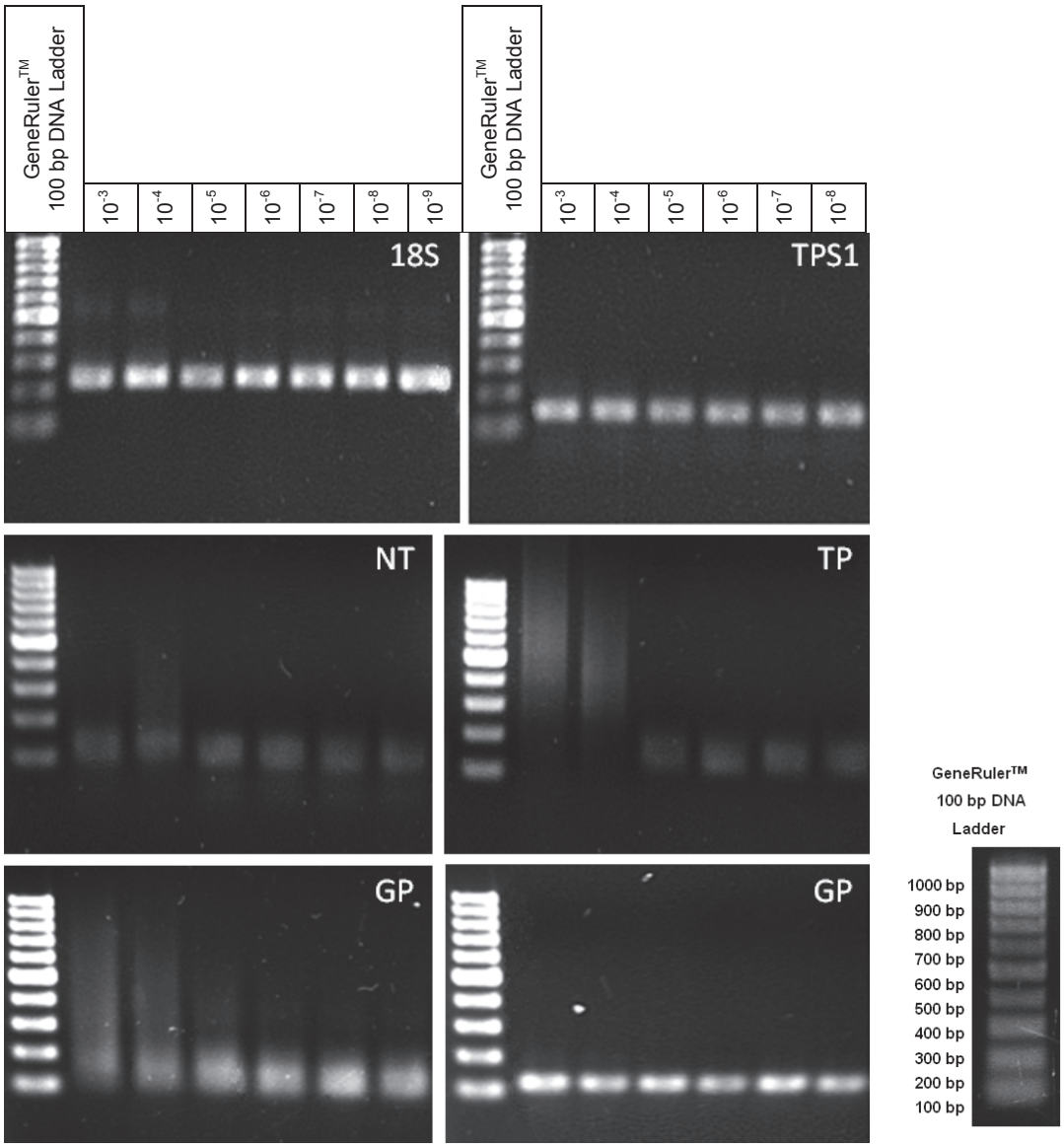


Fig. 50. Dilution series of template DNA for quality analysis of amplification conditions
PCR fragments of the dilution series (10^{-3} to 10^{-8}; 18S up to 10^{-9}) were gel-electrophoretical separated and stained by ethidium bromide. The decreasing concentrations were pipetted into the gel, starting on the left with highest concentration, respectively. Expected fragment sizes were 231 bp (18S rRNA), 129 bp (TPS1), 115 bp (NT), 122 bp (TP), 132 bp (GP), and 136 bp (GS). Each in the first lane GeneRuler™ 100 bp DNA Ladder was loaded.

3.6.4.2 Impact of RNAi-based transcript silencing on gene expression

3.6.4.2.1 Optimisation of RNA isolation and cDNA synthesis

Due to its clear growth-related phenotype and time constraints the main focus was drawn towards *TPS1*-silenced transformants. RNA isolation and also determination of metabolite content was performed using agar plate-based mycelium. For RNA isolation

a kit-based strategy (see 2.5.1.1) was performed, yielding between 20 ng/µL and 300 ng/µL DNA-free total RNA per sample (Fig. 51).

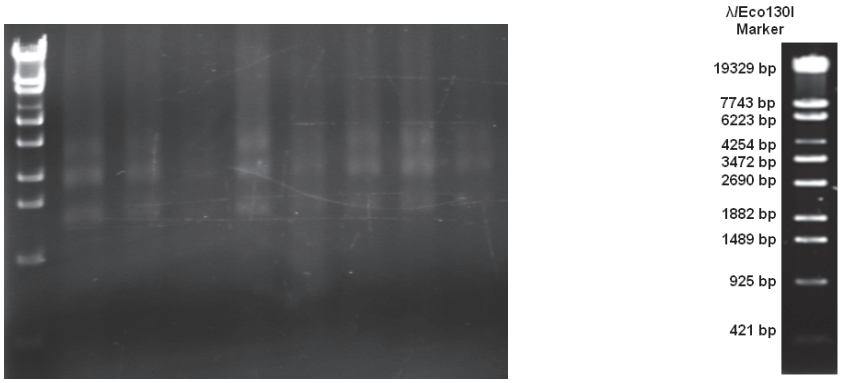


Fig. 51. Quality analysis of total RNA by gel-electrophoresis
Aliquots of isolated total RNA were applied to denaturising agarose gel-electrophoresis. The presence of distinct ribosomal RNA fragments indicated whether RNA was degraded during preparation.

RNA quality was determined by means of denaturing agarose gel electrophoresis as well as qPCR using fungus-specific 18S rRNA primer pairs. As no other calibration marker has been yet developed for *L. bicolor*, the mRNA content in different samples was standardised to 18S rRNA. Different RNA amounts were tested for reproducible cDNA synthesis. A starting amount of 200 ng total RNA turned out to give highly robust reproducible results. Thus, similar RNA amounts were used for cDNA synthesis; CP values of 18S rRNA amplification were expected to be similar, too. RNA preparations giving rise to larger differences from common CP values (which were rare) were thus withdrawn from analysis.

3.6.4.2.2 Modulation of gene expression upon *Laccaria* transformation with a TPS1-RNAi construct

The TPS1-RNAi cassette was introduced into both monokaryotic *L. bicolor* strains S238N-H70 and S238N-H82. As the two monokaryons revealed different growth properties (growth speed, hyphal density), gene expression of *TPS1*, *NT*, *TP*, *GS*, and *GP* was compared for both parental strains for all three harvested fungal age groups (Fig. 52).

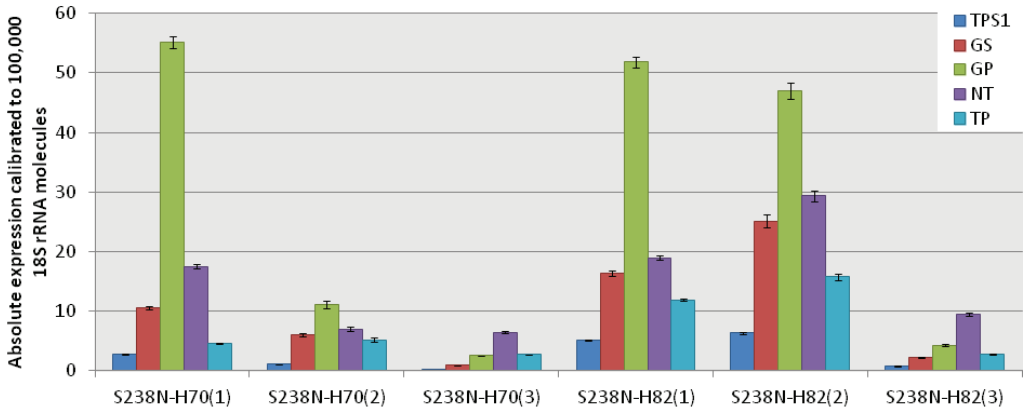


Fig. 52. Expression of *TPS1*, *GS*, *GP*, *NT*, and *TP* genes in both wild type monokaryons
The transcript levels (calibrated to 100,000 18S rRNA molecules) of five genes coding for TPS1 (trehalose-6-phosphate synthase 1), GS (glycogen synthase), GP (glycogen phosphorylase), NT (neutral trehalase), and TP (trehalose phosphorylase) are shown. The parts of the fungal colony are indicated by the numbers in brackets; (1): outermost part of the colony (up to 7 days old hyphae), (2): the middle part (8-14 days old hyphae), (3): central part (15-21 days old hyphae).

While genes coding for *TPS1*, *GS,* and *GP* constantly declined with hyphal age for strain S238N-H70, this was only the case for the oldest part of the fungal colony for strain S238N-H82. Generally gene expression was lowest in the oldest part of the fungal colony for both fungal strains. However, one exception was determined for strain S238N-H70, the *NT* revealed similar transcript levels in the middle and central area.

To analyse the impact of the TPS1-RNAi construct on the expression of trehalose and glycogen metabolic genes, six transformants with S238N-H70 background (TPS1-7003, 4, 6, 7, 8, and 10) and five transformants with S238N-H82 background (TPS1-8204, 6, 7, 8, and 10) were selected. Gene expression was analysed separately for the different parts of the fungal colony (see Fig. 53 for *TPS1*, *NT*, *TP*, and Fig. 54 for *GS* and *GP* data).

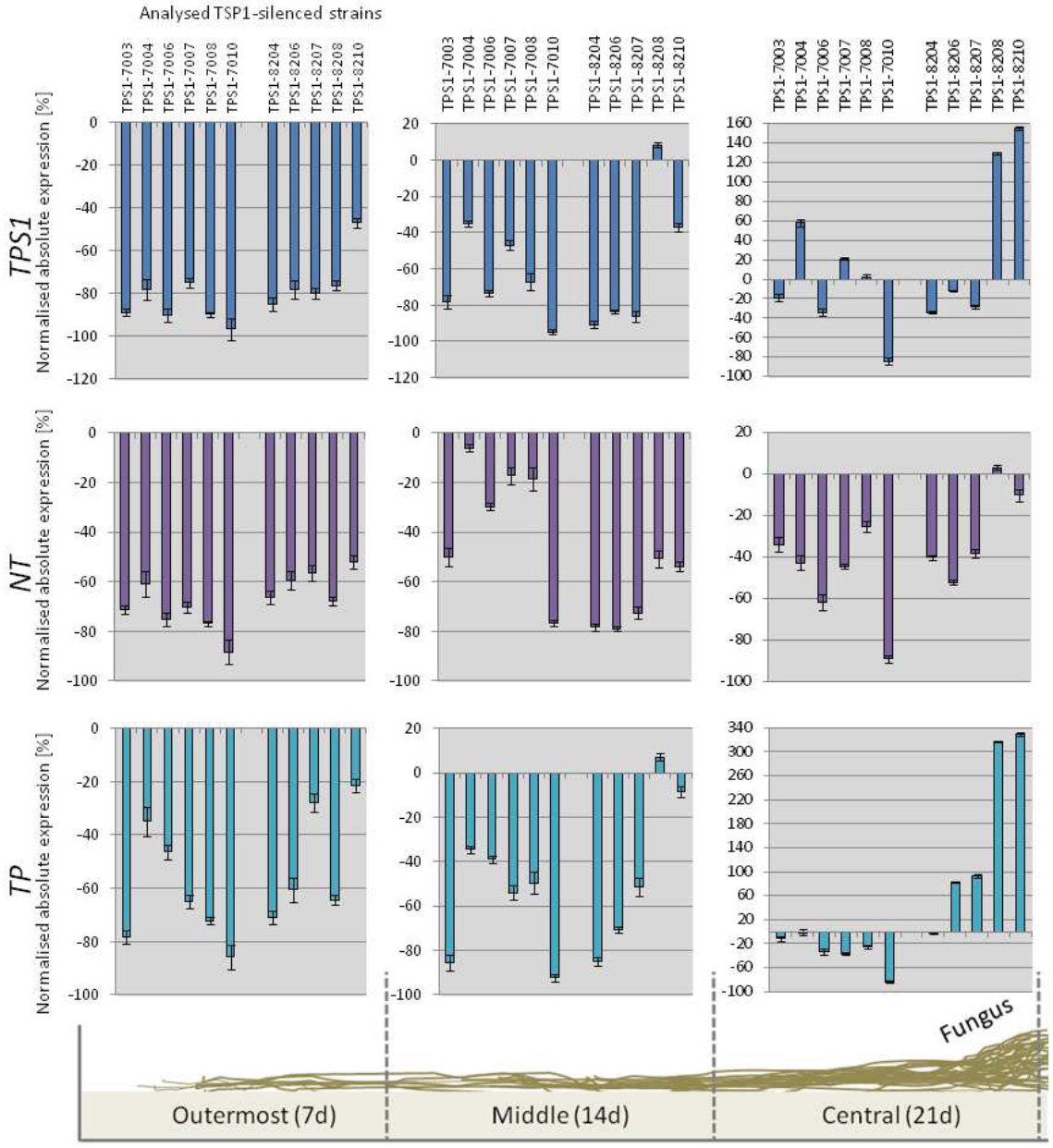


Fig. 53. Gene expression profiles of *TPS1*, *NT*, and *TP* in different parts of the fungal colony
CP levels were calibrated to 100,000 RNA content by 18S rRNA and adjusted to wild type gene expression. Transcript levels are shown as percentage (y-axis) of that of the respective wild type (S238N-H70 or S238N-H82). Always the six bars on the left of each diagram indicate data of S238N-H70 mutants, while the five bars on the right demonstrate the data of S238N-H82 mutants, drawn on the x-axis. All expression differences were proven as statistically mainly highly significant (p-value < 0.01), see appendix Tab. 24.

Interestingly, expression of the TPS1-RNAi construct did not only reduce *TPS1* transcript levels, however, it caused also a severe reduction of gene expression of all investigated genes involved in trehalose and glycogen metabolism. This effect was most pronounced in the youngest part of the fungal colony, similar but frequently less strong in the middle part, but (with exception of *TP*) less severe, invisible or even

reversed in the oldest part of the colony. In contrast to growth behaviour, gene suppression was similar for transformants with S238N-H70 and S238N-H82 background.

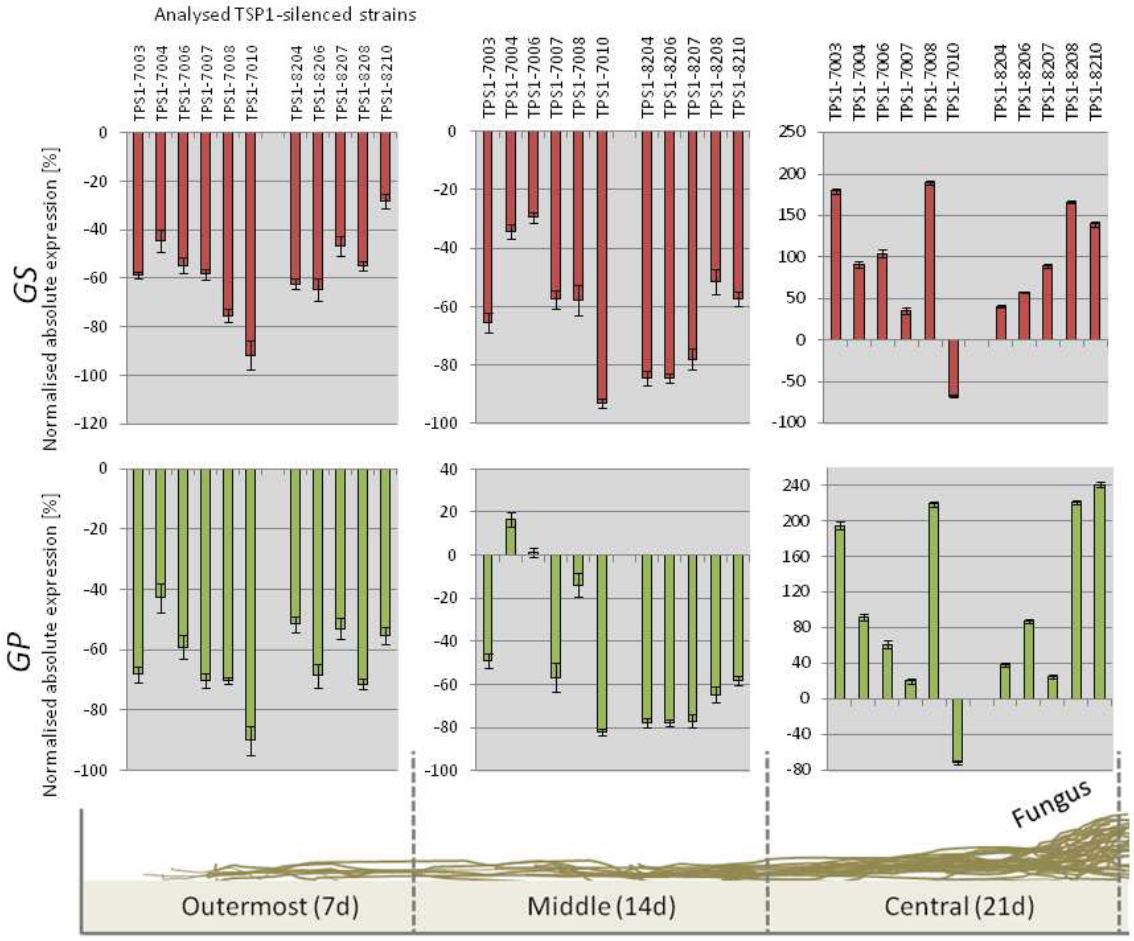


Fig. 54. Gene expression profiles of *GS* and *GP* in different parts of the fungal colony
CP levels were calibrated to 100,000 18S rRNA molecules and adjusted to wild type gene expression. Transcript levels are shown as percentage (y-axis) of that of the respective wild type (S238N-H70 or S238N-H82). Always the six bars on the left of each diagram indicate data of S238N-H70 mutants, while the five bars on the right demonstrate the data of S238N-H82 mutants, drawn on the x-axis. All expression differences were proven as statistically at minimum significant (p-value < 0.05) except for the *GP* expression of TPS1-7006 in the middle sector (not significant), see appendix Tab. 24.

3.6.5 Temperature stress assay

Next to its function in carbohydrate storage, trehalose was described in literature as protectant to hamper temperature stress. To study such trehalose function in *Laccaria bicolor*, growth of fungal hyphae on agar plates was compared for transgenic and non-transgenic strains at different temperatures. For a better visualisation of differences in

growth behaviour, the maximal growth speeds of the transgenic strains was normalised to that of the respective parental strain (Fig. 55).

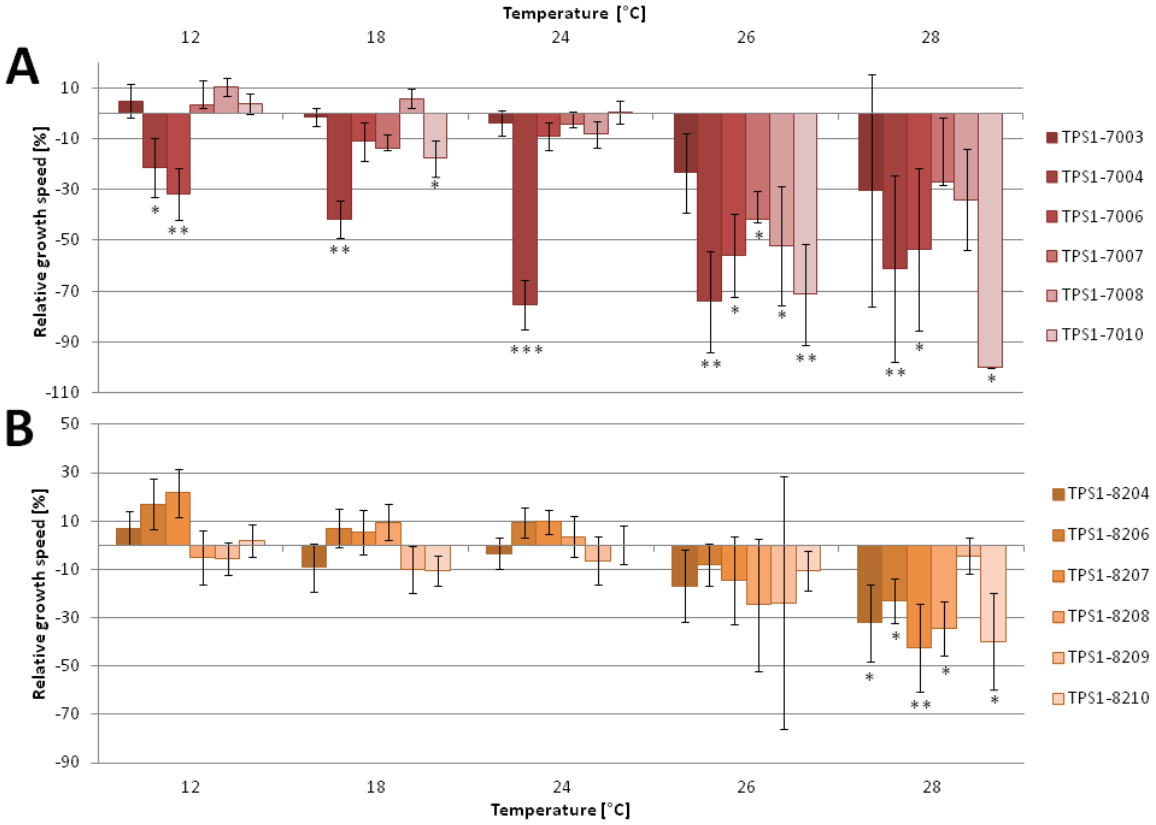


Fig. 55. Relative maximal growth speed of TPS1-RNAi transformants
Transformants and wild type mycelia were exposed to different temperatures and the maximal growth speed was determined. To get a clear picture, the achieved growth speed of the transformants (x-axis) was further normalised to that of the respective wild type (displayed as relative growth speed in % on the y-axis). H70 derivates are indicated in A, while H82 transformants are figured in B. P-values (eight biological replicates were performed) for transgene fungi and wild type strains are indicated by stars (* significant, p-value < 0.05; ** highly significant, p-value < 0.01, and *** extremely significant, p-value < 0.001), or in some cases as not significant indicated with no stars (p-value > 0.05).

With exception to TPS1-7004 and TPS1-7006 the growth rate of all transformants with a S238N-H70 background was similar to that of the parental strain at 12 °C, while at temperatures between 18 and 24 °C only the growth of TPS1-7004 differed noticeable. However, temperatures above 24 °C (a temperature that turned out to be at the border of heat stress for wild type hyphae, too) resulted in an unambiguous strong decrease in hyphal growth rate for all TPS1 transformants (Fig. 55 A). Furthermore, all transformants (except TPS1-7010 where the mycelium quickly died) revealed a strong change in colony morphology at 28 °C, which was only slightly detected in S238N-H70 wild type. The mycelial front revealed a strong sectoral growth, where very fast growing

hyphae frequently started to establish a very thin hyphal layer, causing the massive standard deviations visible in Fig. 55 A.

In contrast, transformants with a S238N-H82 background revealed no obvious differences in their maximal growth speed to that of the wild type for temperatures between 12 and 24 °C. At 26 °C a tendency towards reduction and at 28 °C a significant reduction of the maximal growth speed became visible (Fig. 55 B).

Because of the strongly reduced hyphal growth rates, incubation at 28 °C was prolonged to a period of 16 weeks and photos were taken to document the phenotype. All TPS1-RNAi transformants with a S238N-H70 background revealed brownish coloured mycelium in the centre of the colony (Fig. 56 A) from which very thin and frequently sectoral growing hyphae rose. In contrast, hyphae of the parental strain revealed an uniform mycelium throughout the entire colony.

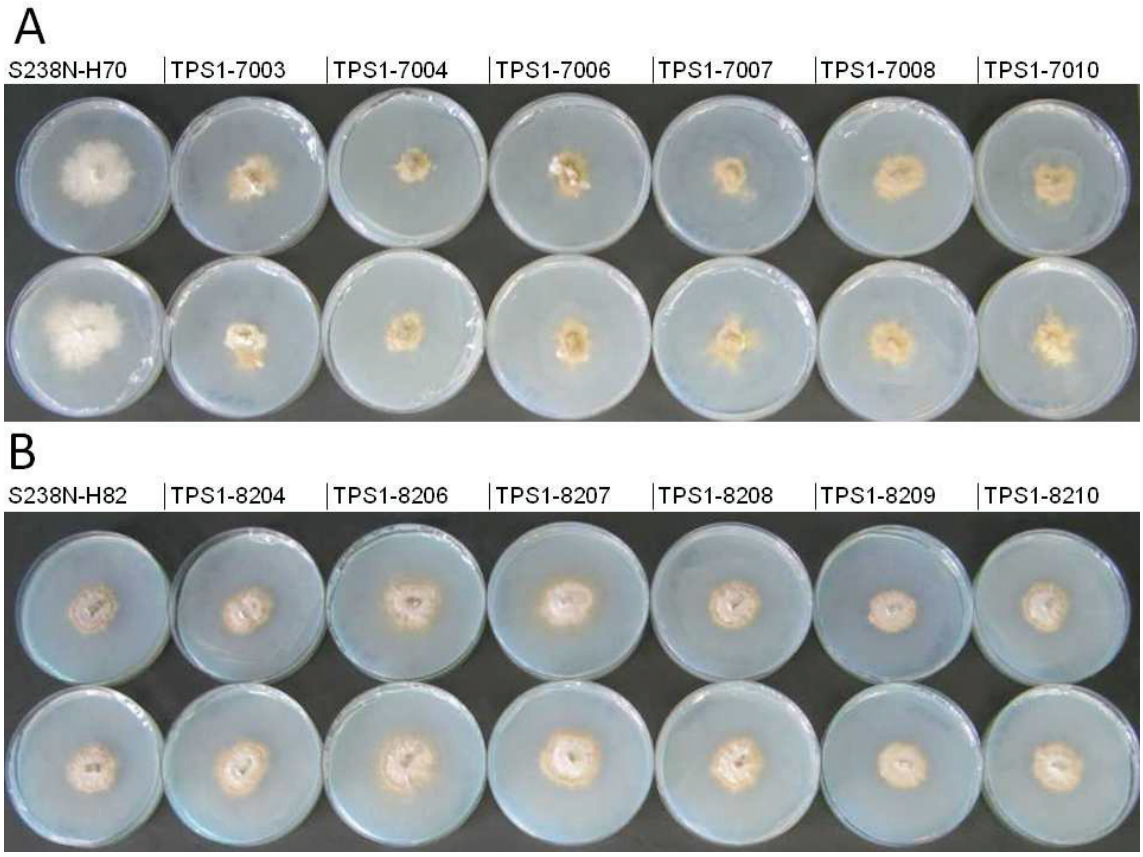


Fig. 56. *TPS1*-silenced mutants and parental strains after 16 weeks at 28 °C
Monokaryotic strains H70, H82, and corresponding silenced strains were exposed to 28 °C for 16 weeks. Afterwards all of the H70-silenced strains were death (brownish coloured and no longer hydrophobic), while the H70 wild type was still alive (A). Also, the strains of H82 background (inclusive wild type) were still alive with more or less growth inhibition. They still looked white and hydrophobic (B). All strains were further tested for their fitness by transferring a hyphal part onto a new MMN plate, and the visible pattern was confirmed. Only the H82 strains, both wild type and transgene, and the H70 wild type restored a healthy colony on the MMN plates at 18 °C.

To avoid drought stress at elevated temperatures the petri dishes were filled with a larger volume of agarose medium and were covered by Parafilm® M (Pecheney Plastics Packaging Inc., Chicago, Illinois, United States). Similar to the transformants also the parental strain revealed brownish coloured mycelium in colonies with a S238N-H82 background (Fig. 56 B). However, at 26 °C colonies seemed to be under physical tension. This phenomenon was most obvious for the transformants TPS1-8206 and TPS1-8207 (Fig. 57).

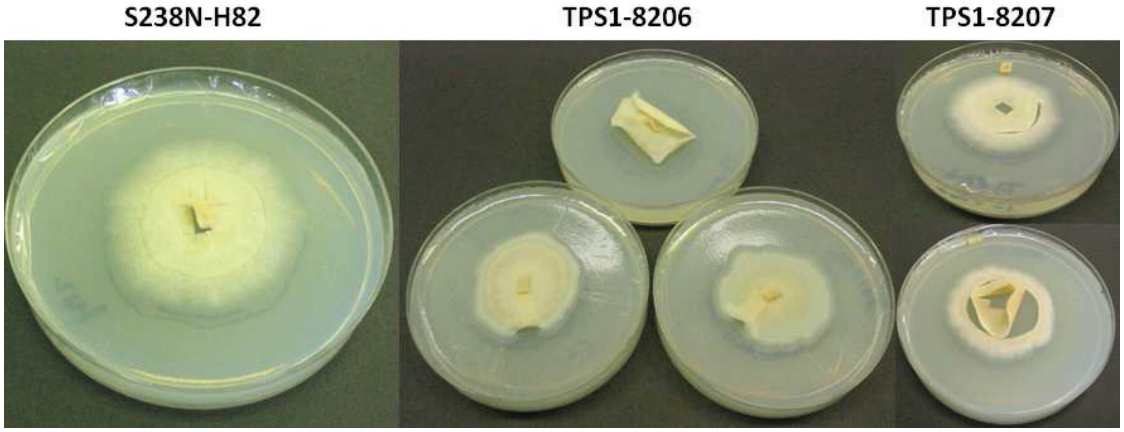


Fig. 57. Phenotypes of H82-TPS1 strains at temperature of 26 °C
Shown are colonies of S238N-H82 wild type (left side), TPS1-8206 (middle), and TPS1-8207 (right side) grown at 26 °C.

3.7 Dikaryon formation

As S238N-H70 and S238N-H82 contain compatible mating type genes, generation of dikaryons was feasible. Therefore, transformants with respective RNAi constructs were simultaneously generated for both strains. For dikaryon formation by induced plasmogamy, confrontation agar plates were established. As only one dominant selection marker is established for *Laccaria* transformation, it could not be used to select for dikaryotic mycelia. Therefore, strain-specific growth properties of S238N-H70 and S238N-H82 were used for dikaryon selection (see below).

All selected combinations of monokaryotic transformants were grown on cellophane desk-covered confrontation plates containing MMN supplemented with 25 mM glucose. After approximately 30 days of co-cultivation, when the growing hyphal fronts of compatible strains met (Fig. 58), a small stripe (about 3 mm x 15 mm in size) from this

confrontation zone was excised (Fig. 18) and transferred onto agar plates for dikaryon selection. The transfer was facilitated by the underlying cellophane desk.

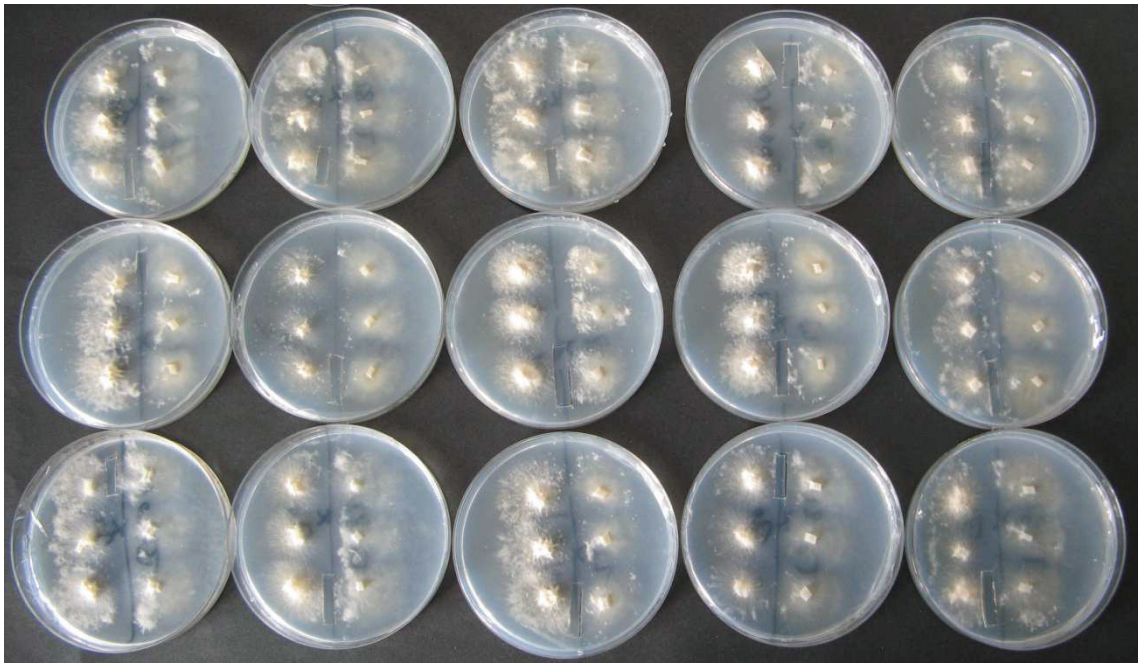


Fig. 58. Confrontation agar plates containing always two *Laccaria bicolor* compatible transformants
MMN agar plates were inoculated with mycelia of compatible monokaryotic transformants. Three agar pieces were used for each transformant. The petri dishes were incubated at 18 °C until the hyphal growth fronts of both transformants got in contact. Thereafter, small stripes of cellophane, carrying the confrontation section, were excised using a sterile scalpel and transferred onto dikaryon selection agar plates.

The selection strategy for dikaryotic hyphae in a monokaryotic background was thus to transfer mycelia of the confrontation zone onto agar plates containing elevated ammonium phosphate concentrations. Monokaryotic *Laccaria bicolor* strain S238N-H82 turned out to be much more susceptible to high ammonium-phosphate concentrations than all other investigated strains, while S238N-H70 revealed massive formation of aerial hyphae (Fig. 59). Furthermore, in contrast to the monokaryotic strain S238N-H70, the mycelia of both dikaryotic strains (S238N, S238N-H82xH70) revealed dark purple coloration at high (10 mM) ammonium phosphate concentrations (Fig. 59).

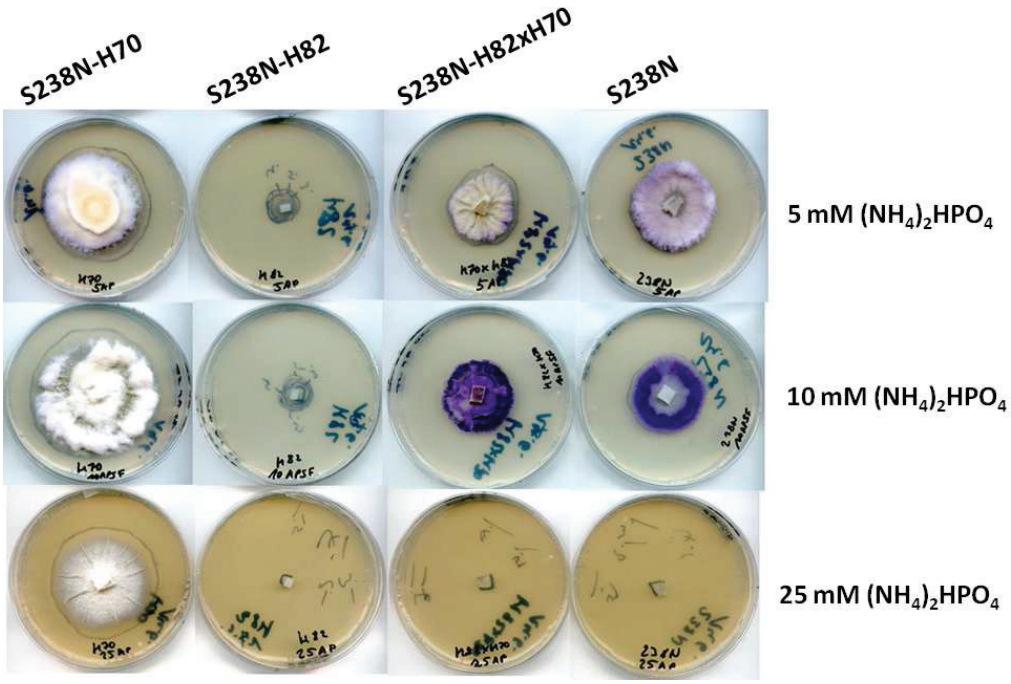


Fig. 59. Phenotypical behaviour of *Laccaria* strains at elevated $(NH_4)_2HPO_4$ concentrations
Shown is the growth behaviour of *Laccaria* strains at three different ammonium phosphate concentrations (5 mM (upper row) 10 mM (middle row), and 25 mM (lower row)). From the left to the right: the monokaryons S238N-H70 (first column) and S238N-H82 (second column), and the dikaryons S238N-H82xH70 (third column) and S238N (fourth column). Photos were taken after 5 weeks of growth.

When fungal mycelia were directly exposed to $(NH_4)_2HPO_4$ concentration of 25 mM, all strains except S238N-H70 died (Fig. 24 and 59). However, dikaryotic mycelia of the confrontation front turned out to be more resistant to elevated ammonium phosphate concentrations. Therefore, finally a concentration of 25 mM $(NH_4)_2HPO_4$ was chosen because of its higher selectivity against monokaryotic mycelia. At this ammonium phosphate concentration, dikaryons did not form the deep purple colour any more.
After transfer of the confrontation zone onto selection agar plates, the initial hyphal growth was frequently sectoral (Fig. 60).

Fig. 60. Growth behaviour of fungal mycelia of the confrontation front on selective agar medium
Stripes of the confrontation front were placed about 2 cm far from the border of a petri dish containing MMN medium supplemented with 25 mM glucose and 25 mM $(NH_4)_2HPO_4$. Large amounts of white aerial mycelia indicate presumably S238N-H70 monokaryons.

Pieces of the mycelial front of fast growing, non fluffy mycelia were excised with a scalpel and transferred onto new selection agar plates (for exact numbers of achieved potentially dikaryotic fronts per cross, see appendix Tab. 26). This procedure was repeated until homogeneous colony-formation was obtained. After two to maximal three growth cycles on selective agar plates, most of the isolates turned out to be dikaryons (see below).

3.7.1.1 Fluorescence microscopic identification of dikaryotic fungi

Putative dikaryotic fungal mycelia were inspected by light and fluorescence microscopy to confirm their dikaryotic status. Typical structures of such dikaryotic mycelia are clamp connections (see introduction section).

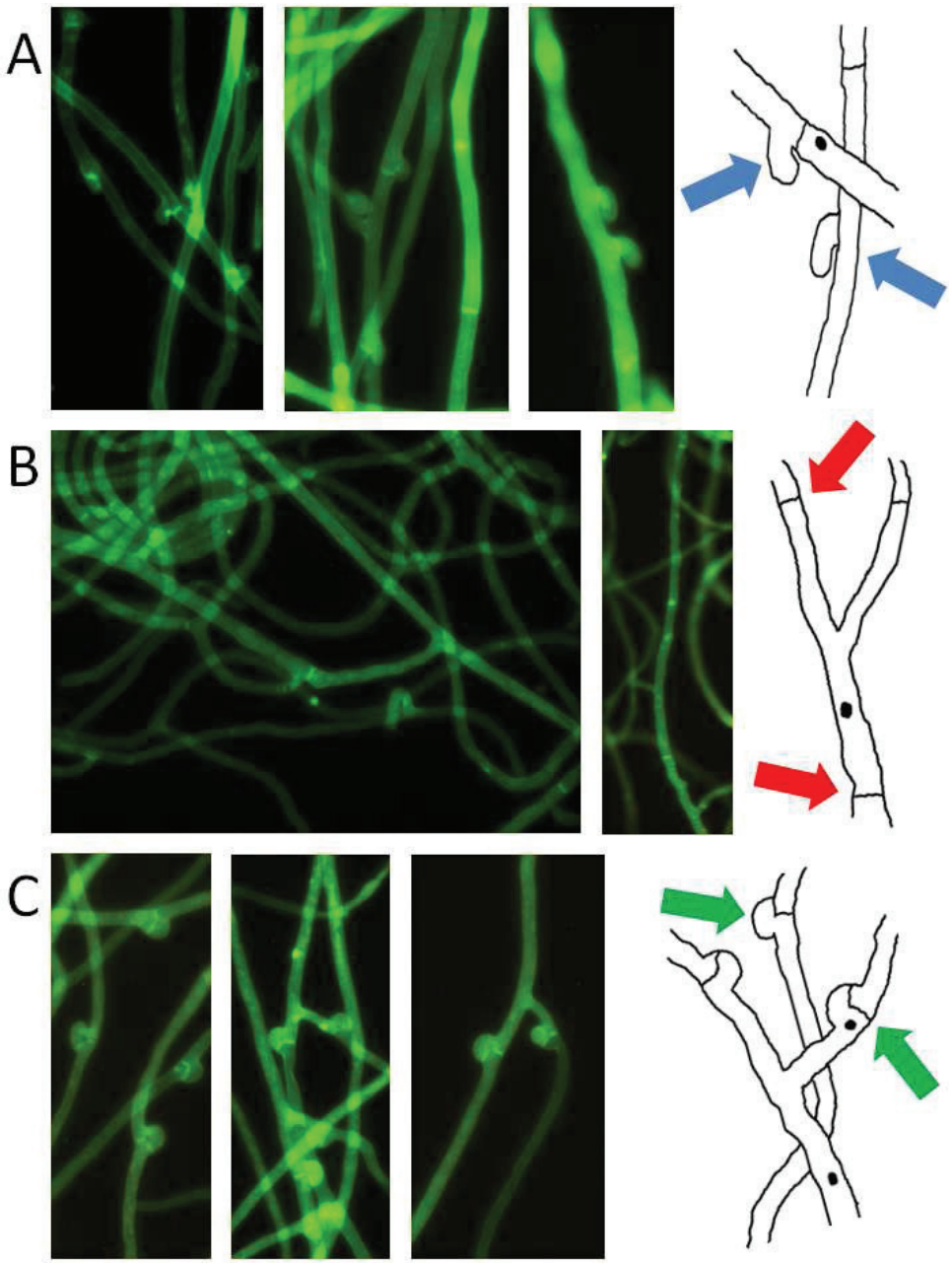


Fig. 61. Typical hyphae of both monokaryotic and one dikaryotic *Laccaria bicolor* strains
Hyphal cell walls were stained with Calcofluor White and visualised by UV light (340-380 nm for Calcofluor White excitation and 430 nm as filtered emission for detection). Pictures were taken with 400-fold magnification. In A, the hyphal structure of monokaryon H70 is pictured, characterised by unfused or pseudo clamp cells, while the hyphae in B display the pattern of monokaryon H82 with normal formed septa and absence of clamp cells. Typical dikaryotic hyphae are figured in C in which the stained hyphae of 238N are represented, indicated by fused clamp cells. In the schematic views the red arrows indicates normal septa between hyphal cells, the blue arrows a pseudo clamp, and the green arrows a real clamp connection; the black dots highlight potential visible nuclei.

Certain monokaryons do form incomplete, so-called pseudo clamp connection (S238N-H70, see Fig. 61 A), short hyphal branches that do not fuse with the underlying main hyphae. Other monokaryon do not form any pseudo clamps (S238N-H82, Fig. 61 B).

Missing or pseudo clamp connections can, however, be easily distinguished from real clamp connections by light microscopy. Furthermore, for better visualisation, fungal cell walls are typically stained with Calcofluor White and inspected by fluorescence microscopy. Pictures of both monokaryotic strains and one dikaryotic strain (Fig. 61 C) are shown to demonstrate typical differences.
In Fig. 62 real clamp connections containing hyphae of eight (confirmed) dikaryons, harbouring different RNAi constructs (NT and TPS1), are exemplarily pictured.

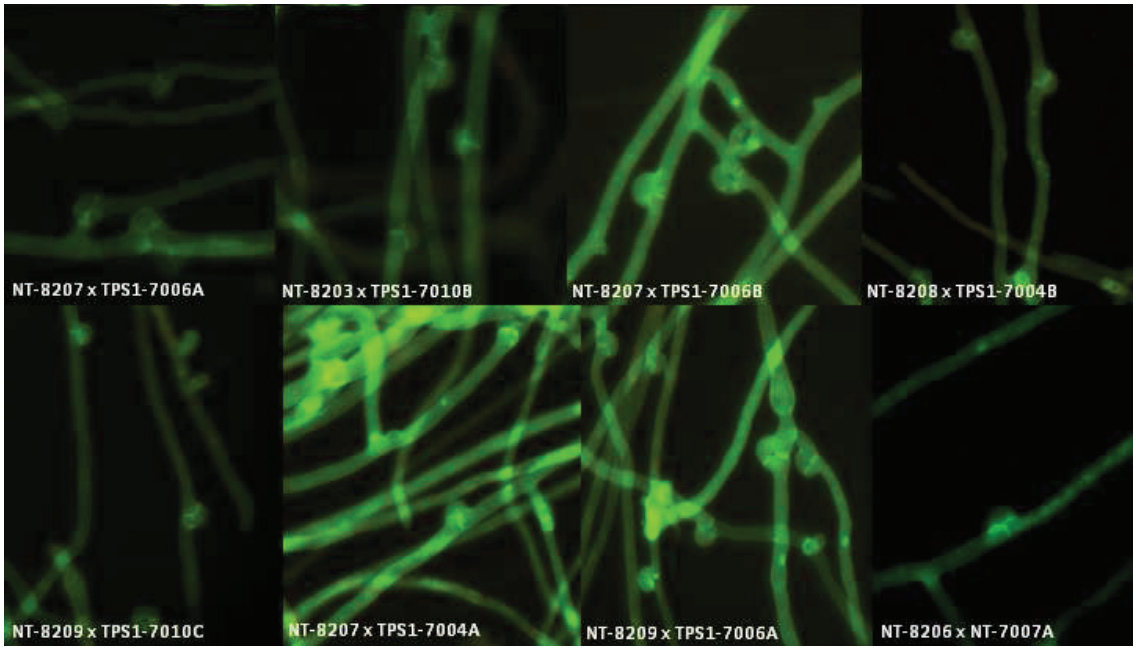


Fig. 62. Selected dikaryons resulting from hyphal fusions of different RNAi construct containing transformants
Hyphal cell walls were stained with Calcofluor White and visualised by UV light (340-380 nm for Calcofluor White excitation and 430 nm as filtered emission for detection). Pictures were taken with 400-fold magnification.

Finally, out of a total of 53 isolated putative dikaryons, only three did not reveal real clamp connections, indicating them as monokaryons (detailed results can be followed in appendix Tab. 26). Therefore, the dikaryon isolation strategy that has been developed in this work, turned out to be rather efficient.

4 Discussion

4.1 Selection of target genes for manipulation of trehalose metabolism

Aim of the work was the manipulation of fungal carbohydrate storage metabolism. Therefore, genes coding for proteins involved in trehalose and glycogen biosynthesis and degradation were identified, confirming and even expanding the released scheme of Deveau (2008).

Genes coding for trehalose phosphorylase, trehalose-6-phosphate phosphatase, and trehalose-6-phosphate synthase are present in the *Laccaria* genome only in single copies. For trehalose-6-phosphate synthase two additional but highly truncated copies were observed, too. Three genes coding for putative neutral trehalases of which one revealed (ID 297216) higher homology to functionally characterised neutral trehalases of other fungi are present in the genome of *Laccaria*. Regarding the glycogen metabolism, only a single glycogen synthase and a single potential glycogenin were identified encoded in the *Laccaria* genome, while yeast encodes each two. Furthermore, single copy genes were identified coding for a potential GLC3 (glycogen branching enzyme) and a SGA1 (glycogen degrading glucoamylase), while three possible GDB1-like (debranching enzyme) protein coding genes were obtained of which especially the ID 302750 shared high homology to yeast. The other two GDB1-like proteins are truncated versions, regarding their short amino acid sequences of each 115 aa; the native yeast GDB1 comprises 1536 aa. As glycogen phosphorylase again a single coding gene was discovered in the database.

4.2 Growth behaviour of *Laccaria bicolor*

Studies with other fungi revealed a strong impact of manipulation of trehalose metabolism on fungal growth behaviour (Foster, Jenkinson et al. 2003; Doehlemann, Berndt et al. 2006; Wilson, Jenkinson et al. 2007; Ngamskulrungroj, Himmelreich et al. 2009; Puttikamonkul, Willger et al. 2010; Fernandez and Wilson 2011). Furthermore, fungal growth conditions are known to modulate the strength of a given antibiotic-based selection pressure. Finally, with regard of their growth behaviour, barely characterised *Laccaria* strains were used and extensive analysis of hyphal growth behaviour was carried out in this work.

Thus, two approaches were performed, determination of maximal growth speed of the hyphal front and hyphal density. Comparison of the maximal growth speed allows a first impression on fungal tip growth, which is important for colonisation of new territories. Hyphal density, however, reflects the branching behaviour of hyphae, which is important for lasting nutrient exploration at the front of the growing colony. Furthermore, in older parts of the colony increasing hyphal density is connected to elevated formation of aerial hyphae, frequently indicating initiation of fruiting body formation (which was never observed on petri dishes with *Laccaria*). Especially on elevated ammonium, the amount of soil born mycelium was strongly enhanced, potentially indicating a dependency of nitrogen and initiation fruiting.

Finally, growth speeds of the assayed *Laccaria* wild type strains seem to be further controlled by seasonal circumstances, because maximal growth speeds were different in summer (heat stress assay), spring (density and growth speed determination), and autumn (glucose, ammonium, and selection marker tests). This possibly matches to the common occurrence of ectomycorrhizal fruiting bodies in autumn, indication a lifecycle interconnected with the season.

4.2.1 Antibiotics

Currently, only one dominant selection marker (hygromycin resistance) is used for *Laccaria* transformation (Kemppainen, Circosta et al. 2005; Kemppainen, Duplessis et al. 2009). However, for certain approaches (e.g. dikaryon formation) more than one selection system is needed. A screen of antibiotics successfully used for selection in other fungi (carboxin, (Honda, Matsuyama et al. 2000; Costa, Thomas et al. 2009; Ngari, Combier et al. 2009); kanamycin, (Costa, Thomas et al. 2009); BASTA, (Zhang, Hampp et al. 2005)) revealed carboxin as potential suitable marker for Laccaria transformation, being toxic at concentrations above 5 µg/mL for all tested *Laccaria* strains. However, in prior studies it was demonstrated that the carboxin conferring resistance cassette differs in functionality, potentially depending on the genetic background of the chosen target fungus. As a *Pleurotus* variant (Honda, Matsuyama et al. 2000) was not functional in *Hebeloma* (Ngari, Combier et al. 2009). Thus, and because of that the resistance cassette could be obtained just shortly before the end of the thesis, the resistance cassette was not transformed into *Laccaria*.

In contrast, the herbicide BASTA revealed hyphal background growth that could not be abolished even by high concentrations. In plant transformation, BASTA sensitivity could be increased by ammonium addition. However, this approach was not successful with any of the *Laccaria* strains. Nevertheless, this experiment revealed strong phenotypical

differences in the growth behaviour of mono- and dikaryotic *Laccaria* strains, leading to a tool that could be used for dikaryon selection (see below).

4.3 Differences between monokaryons

Strong differences were already described for certain monokaryotic *Laccaria*, for instance in their acid phosphatase activity (Kropp 1990; Lumley, Farquhar et al. 1995), and for *Hebeloma* monokaryons, regarding nitrate reductase and glutamate dehydrogenase activity (Debaud, Gay et al. 1988). Profound differences between both investigated monokaryotic *Laccaria* strains S238N-H70 and S238N-H82 were also obtained in this work. S238N-H82 revealed a significant lower maximal growth speed at the optimal temperature range between 18 and 24 °C. Here, also hyphal density was lower at the hyphal front as well as in the oldest part of the fungal colony. In contrast, hyphal density was similar for S238N-H70 and S238N-H82 in the middle parts of the colonies. Lower hyphal density at the colony front, but similar density in the older (middle) part of the colony, indicated that efficient nutrient exploration (hyphal branching) by S238N-H82 takes place later than in the case of S238N-H70. S238N-H82 is also much more sensitive to elevated ammonium phosphate levels. Since phosphate did not reveal such strong growth suppression at comparable concentrations (data not shown) and ammonium chloride suppressed hyphal growth of all investigated strains in a similar manner, the combined presence of elevated ammonium and phosphate concentrations cause the observed phenotypical effects.
At elevated (28 °C) temperature, however, S238N-H82 could resist heat for a longer time period and revealed a similar maximal growth speed as S238N-H70. Also at lower (12 °C) temperature, maximal growth speed was similar for both monokaryons, together indicating towards a higher stress tolerance. One reason for this might be the higher carbohydrate content in all parts of the colony of S238N-H82 (Fig. 63, (Felmeth 2012; Samborski 2012). Especially increased trehalose content is often associated with higher heat stress resistance (Eleutherio, Araujo et al. 1993; Arguelles 1994; De Virgilio, Hottiger et al. 1994; Arguelles 1997; Lewis, Learmonth et al. 1997; Soto, Fernandez et al. 1999; Fillinger, Chaveroche et al. 2001; Doehlemann, Berndt et al. 2006; Li, Ye et al. 2009; Al-Bader, Vanier et al. 2010; Salmerón-Santiago, Pardo et al. 2011).

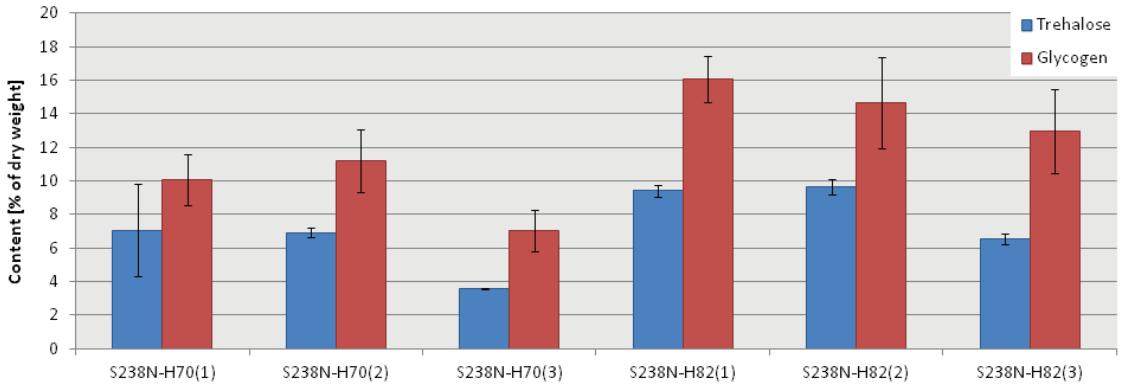


Fig. 63. Trehalose and glycogen content of monokaryotic *Laccaria* strains
Fungal mycelia were grown on agar plates at 18 °C and colonies were dissected according to Fig. 42. Trehalose (blue bars) and glycogen (red bars) content were determined for the *Laccaria* monokaryons S238N-H70 and S238N-H82. Displayed are the carbohydrate amounts in percentage of total mycelial dry weight (data from Felmeth 2012, Samborski 2012). The numbers in brackets behind the strain name indicate the growth zone, as 1 indicates the 7 days old hyphal front, 2 the middle part of the fungal colonies (seven to 14 days old), and 3 the oldest part of the colony (aged up to 21 days).

Trehalose content in the different parts of the fungal colony correlated very well with gene expression of *TPS1* for both monokaryotic strains. This matches data from literature (*Cryptococcus* (Petzold, Himmelreich et al. 2006), and *Aspergillus* (Wolschek and Kubicek 1997), where *TPS1* expression was strongly induced when hyphae were exposed to a good carbohydrate source. Same pattern was revealed for the glycogen content and the *GS* expression in *Laccaria*. In *Saccharomyces*, induced transcription of *TPS1* and of both glycogen synthases (*GSY1*, *GSY2*) can also be correlated with increased trehalose and glycogen contents. Furthermore, the degradative *NT* (*NTH1)* and *GP* (*GPH1*) were also induced in chorus with the synthetic enzymes in yeast (Zahringer, Holzer et al. 1998; Parrou, Enjalbert et al. 1999), as here also detected in *Laccaria*. As the degradative protein coding genes (*NT*, *TP*, and *GP*) revealed correlation of transcript levels and metabolite contents. This is, however, not surprising as the respective enzymes are highly regulated by posttranscriptional regulation (phosphorylation, see discussion below).

A striking difference between the two monokaryons was the ability of S238N-H70 to form pseudo clamps. This indicates that in S238N-H70 *A*-type mating gene encoded proteins are much more active than in S238N-H82. Pseudo clamp formation and *A*-type gene encoded proteins are intensively discussed in fungal literature (Kothe 2001; Raudaskoski and Kothe 2010; Yi, Mukaiyama et al. 2010). When co-incubated with a poplar as host plant for a longer period (at least 3 month), hyphae, attached to the root surface and starting to amplify next to the root, were observed for S238N-H70 but not

for S238N-H82 (data not shown). Root attachment is the first step towards host root colonisation in ectomycorrhizal symbiosis. In nature, ectomycorrhizae are formed usually by dikaryotic fungal hyphae. However, in culture monokaryotic strains of certain model fungi have been shown to be capable of forming mycorrhizae, too (e.g. *Laccaria, Hebeloma,* and *Pisolithus* (Kropp, McAfee et al. 1987; Kropp and Fortin 1988; Wong, Piché et al. 1989; Kropp 1990; Wong, Piché et al. 1990; Burgess, Dell et al. 1994; Di Battista, Selosse et al. 1996; Silva, Costa et al. 2007; Costa, Campos et al. 2010). Thus, a future question will be, whether the capability to form pseudo clamps and to form mycorrhiza or mycorrhiza initials coincide. This can be a hint for a correlation of mating type gene expression and mycorrhiza formation.

4.4 *Agrobacterium*-based transformation of *Laccaria bicolor*

First attempts of genetic transformation of fungi by agrobacterial infection were successfully performed with *Saccharomyces cerevisiae* (Bundock, den Dulk-Ras et al. 1995; Bundock and Hooykaas 1996; Piers, Heath et al. 1996), followed by a number of ascomycotic filamentous fungi like *Aspergillus awamori*, *A. niger, Fusarium venenatum, Trichoderma reesei, Colletotrichum gloeosporioides,* and *Neurospora crassa* (de Groot, Bundock et al. 1998; Gouka, Gerk et al. 1999). Today, *Agrobacterium*-based fungal transformation is also feasible for basidiomycetes (*Heterobasidion annosum* (Samils, Elfstrand et al. 2006) *Agaricus bisporus* (van de Rhee, Graca et al. 1996; de Groot, Bundock et al. 1998; Chen, Stone et al. 2000)), including ectomycorrhizal fungi (*Suillus bovinus* (Hanif, Pardo et al. 2002), *Laccaria bicolor* (Kemppainen, Circosta et al. 2005; Kemppainen and Pardo 2011) and *Hebeloma cylindrosporum* (Combier, Melayah et al. 2003; Ngari, Combier et al. 2009)) and became the favoured technique for genetic manipulation of fungi in general.

However, even when previously attempts to perform transformation of mono- and dikaryotic *Laccaria* strains, following published protocols (for instance Kemppainen et al. 2005) and using a commonly applied vector initially failed. Therefore, systematic adaptation of protocols successfully used for fungal and even plant transformation was performed. The effect of different attempts for optimisation of *Laccaria* transformation is discussed below.

4.4.1 Choosing an appropriate agrobacterial strain

The first attempt was the utilisation of different strains of *Agrobacterium tumefaciens* known from plant transformation. Out of three investigated strains AGL1 (Chabaud, de Carvalho-Niebel et al. 2003), C58/PMP90 (Leple, Brasileiro et al. 1992), LBA 4404 (Li,

Li et al. 1999) only AGL1 turned out to be suitable to produce (few) hygromycin resistant colonies in initial experiments. AGL1 is known to be hypervirulent (Jin, Komari et al. 1987; Lazo, Stein et al. 1991) and is thus widely used in plant (Chabaud, de Carvalho-Niebel et al. 2003; Wu, Sparks et al. 2003; Alvarez, Alonso et al. 2004; Wu, Doherty et al. 2008; Alvarez-Fernandez and Ordas 2012), but also in fungal (Chen, Stone et al. 2000; Hanif, Pardo et al. 2002; Combier, Melayah et al. 2003; Grimaldi, de Raaf et al. 2005; Kemppainen, Circosta et al. 2005) transformation.

4.4.2 Pre-culture conditions known to modulate transformation efficiency

T-DNA transfer into the host cell by agrobacteria is mediated by a specific pilus, which is only formed when plant derived acetosyringone is recognised (Fullner, Lara et al. 1996). This phenolic compound is synthesised by plant cells after wounding. Pre-incubation with exogenously applied acetosyringone prior to transformation is thus common for transformation protocols. Acetosyringone induces the expression of virulence genes necessary for plant infection in *Agrobacterium* (Roitsch, Wang et al. 1990; Scheeren-Groot, Rodenburg et al. 1994; Bundock, den Dulk-Ras et al. 1995; Gelvin 2000). Doubling the acetosyringone concentration to 200 µM caused a 100-fold increase in agrobacterial *vir* gene expression (Cangelosi, Ankenbauer et al. 1990; Huang, Cangelosi et al. 1990). In contrast to phenolic compounds from plants, no fungal metabolite is known that have similar effects on agrobacterial gene induction (Bundock, den Dulk-Ras et al. 1995; de Groot, Bundock et al. 1998). Accordingly, fungal transformation seems to be absolute dependent on acetosyringone-based pili induction (de Groot, Bundock et al. 1998; Mullins, Chen et al. 2001; Pardo, Hanif et al. 2002; Combier, Melayah et al. 2003). A pre-induction with 200 µM acetosyringone resulted in strongly increased transformation efficiencies in the different fungal protocols (Bundock, den Dulk-Ras et al. 1995; Chen, Stone et al. 2000; Mullins, Chen et al. 2001), while further increase to 300 µM did not gave rise to higher transformation rates (Chen, Liu et al. 2007). It was also recently publicised for *Laccaria bicolor* transformation (Kemppainen and Pardo 2011), while older transformation protocols (available at the begin of this thesis) did not mention acetosyringone pre-incubation of agrobacteria at all (Pardo, Hanif et al. 2002; Kemppainen, Circosta et al. 2005).

Furthermore, transformation promoting effects were observed when sucrose (between 0.5 and 10 % (w/v)) was added to the co-inoculation medium (Clough and Bent 1998; Davis, Hall et al. 2009). Therefore, sucrose (final concentration of 5 % (w/v)) was

tested as component of the inoculation solution (drooping solution) for *Laccaria* transformation, but no positive effect was determined.

4.4.3 Co-culture conditions known to modulate transformation efficiency

A close contact of agrobacteria and host cell is essential for transformation. Therefore, vacuum infiltration is often used to increase *Agrobacterium*-based plant (Bechtold and Pelletier 1998; Clough and Bent 1998; Tague and Mantis 2006) and fungal transformation (Chen, Stone et al. 2000). When grown on agar plates, fungal colonies are frequently hydrophobic most likely due to hydrophobins (small proteins with the ability to form hydrophobic surfaces) masking the hyphal surface (Wessels, De Vries et al. 1991; Kershaw and Talbot 1998). Vacuum infiltration is thus frequently applied to drive bacteria into the intercellular air space of fungal mycelia, as published for *Agaricus* (Chen, Stone et al. 2000). Presumably due to the hydrophilic character of the hyphal surface, vacuum infiltration did not increase transformation efficiency in *Laccaria.*

Whalen et al. (1991) observed that agrobacterial infection of *Arabidopsis* leaves was significantly increased by application of the surfactant Silwett L-77 (at concentrations between 0.0002 and 0.05 % (v/v)). This silicon-based chemical depresses surface tensions of aqueous solutions and allows droplets to spread evenly over the leaf surface and to penetrate stomatal openings (Whalen, Innes et al. 1991). In the plant transformation, vacuum infiltration of agrobacteria is thus often replaced by Silwett L-77 application (Cheng, Fry et al. 1997; Clough and Bent 1998; Bent 2000; Tague and Mantis 2006; Davis, Hall et al. 2009; Li and Nebenfuhr 2010). Furthermore, 0.05 % (v/v) Silwett L-77 turned out to be non-toxic for hyphal growth of *Laccaria*.

$MgSO_4$ (10 mM) is commonly used in combination with a surfactant in plant infection (Grimsley, Ramos et al. 1988; Shimoda, Toyoda-Yamamoto et al. 1990; Schlappi and Hohn 1992; Shen, Escudero et al. 1993; Gartland and Davey 1995; Aida and Shibata 1996; Shen, Escudero et al. 1999; Alvarez, Alonso et al. 2004) and is highly recommended when inoculating plants with hypervirulent agrobacteria (personal communication Caterina Brancato and Anja Hoffmann, Plant transformation unit of the ZMPB - Centre for Plant Molecular Biology (University of Tübingen, Tübingen, Germany)). Magnesium sulphate appears to reduce aggregation of agrobacteria (Meyers, Chakauya et al. 2008), especially at high densities that are usually used in fungal transformation (see below). The addition of $MgSO_4$ and 0.05 % (v/v) Silwett l-77 leaded to a strong increase in transformation efficiency (almost 7-fold higher).

High agrobacterial densities have been found to increase transformation efficiency for a number of fungi (*Fusarium oxysporum*, *Coccidioides immitis* (Abuodeh, Orbach et al. 2000; Mullins, Chen et al. 2001), *Hebeloma cylindrosporum* (Combier, Melayah et al. 2003)). Therefore, *Laccaria* transformation efficiency was compared using two different agrobacterial densities (OD_{600} of 1 and 5). Higher densities of *Agrobacterium* (in combination with $MgSO_4$ and 0.05 % (v/v) Silwett l-77) resulted in higher transformation efficiency (for up to a factor of four) in most of the experiments.

The addition of 10 mM glucose, arabinose, xylose, and galactose or glucose analogous, or 0.5 % (w/v) sucrose could also enhance *vir* gene induction and plant transformation efficiency (Clough and Bent 1998). A synergistic 200-fold increase in *vir* gene expression was described by combined monosaccharide and acetosyringone application (Cangelosi, Ankenbauer et al. 1990; Shimoda, Toyoda-Yamamoto et al. 1990). For *Laccaria* transformation, different concentrations of glucose, sucrose, and fructose in the co-cultivation medium were tested, of which a combination of 0.5 % (w/v) sucrose with 0.5 % (w/v) glucose resulted in the highest transformation efficiency (more than 16 %).

Temperatures between 19 and 22 °C were discovered to be optimal for agrobacterial pilus formation necessary for the infection of plants (Fullner, Lara et al. 1996; Fullner and Nester 1996; Dillen, De Clercq et al. 1997; Chakrabarty, Viswakarma et al. 2002; De Clercq, Zambre et al. 2002; Li, Shi et al. 2003). Temperature dependency of fungal transformation efficiency was also described. Optimal temperatures for co-cultivation with *Agrobacterium* were 20 °C for *Suillus*, *Hebeloma,* and *Paxillus* (Pardo, Hanif et al. 2002), 23 °C for another *Hebeloma* protocol (Combier, Melayah et al. 2003), and 18 to 24 °C for *Agaricus bisporus* (Chen, Stone et al. 2000; Mikosch, Lavrijssen et al. 2001) and some other filamentous fungi (de Groot, Bundock et al. 1998). Thus, for transformation of *Laccaria* strains temperatures of 18 and 24 °C were tested of which exclusively the elevated temperature was determined as suitable.

Plant transformation efficiency of agrobacteria has also been shown to increase when bacteria from the early stationary growth phase are used (Clough and Bent 1998). Accordingly, for all experiments aiming *Laccaria* transformation, agrobacteria from early exponential growth phase were used. Not only for *Agrobacterium* but also for *Laccaria* the growth phase could be important for transformation efficiency. Accordingly, fungal colonies, aged between 6 (3 mm colony diameter) and 40 days (30 mm colony diameter), were assayed for their transformation capability. A significant preference for younger colonies was discovered as colonies pregrown for almost 10 days (maximal 10 mm colony diameter) revealed highest efficiencies.

The duration of co-cultivation of agrobacteria and *Laccaria* mycelia has an impact as known from fungal and plant transformations (Cervera, Pina et al. 1998; de Groot, Bundock et al. 1998; Pardo, Hanif et al. 2002), revealing a period of about 5 days to being optimal. Exactly 5 days of co-culture were thus chosen for *Laccaria* transformation, too. Furthermore, the appropriate pH value (pH 5.0 to 5.8) during co-culture turned out to be essential for the induction of agrobacterial virulence genes (Stachel, Nester et al. 1986; Vernade, Herrera-Estrella et al. 1988; Mantis and Winans 1992) and t-DNA transfer into the host organism (Bundock, den Dulk-Ras et al. 1995). In natural plant infection, bacteria are stimulated by the characteristic apoplastic acidification upon wounding (Sheng and Citovsky 1996) in combination with acetosyringone (Vernade, Herrera-Estrella et al. 1988). Accordingly, the pH of the co-culture medium was adjusted to pH 5.2 using 10 mM 2-(N-morpholino) ethanesulfonic acid (MES)-KOH buffer for *Laccaria* transformation.

Addition of 1 mM of the bacterial osmo-protectant betaine (Styrvold, Falkenberg et al. 1986) has been demonstrated to induce especially the *virD* genes (Vernade, Herrera-Estrella et al. 1988), which are essential for t-DNA excision and transport into the host nucleus. This betaine effect could be further enhanced by addition of $CaCl_2$ (final concentration of 0.1 mM (Vernade, Herrera-Estrella et al. 1988)). Therefore, the co-inoculation medium during Laccaria transformation was supplemented with 1 mM betaine and/or 0.1 mM $CaCl_2$, however, highest efficiencies of 11.0 and 16.4 % were reached when only betaine was applied.

4.4.4 Selection of fungal transformants

Complete inhibition of hyphal growth has been observed at hygromycin concentrations of 200 µg/mL and above. Therefore a concentration of 300 µg/mL was applied for initial selection of putative *Laccaria* transformants in initial experiments. However, after co-cultivation with agrobacteria, fungal hyphae became somehow resistant to this hygromycin concentration. Growing hyphae were observed after a few weeks of selection on all fungal colonies on the selection plates. Increasing the hygromycin concentration to 500 µg/mL, however, solved this fungal background growth.

4.4.5 Conclusion - transformation

The highest transformation efficiency was reached with almost 16.4 % transformed colonies through an optimised preparation and co-cultivation of both agrobacteria and *Laccaria* hyphae. Thus, different pregrowth conditions (duration, medium, and pre-induction of bacteria) followed by a co-cultivation period on specific transformation petri

dishes supplemented with different carbohydrates (glucose, sucrose, and the sugar alcohol glycerol), an appropriate pH, and different chemicals (to continuously induce the virulence genes of *Agrobacterium*) facilitated the t-DNA transfer. This efficiency was not as high as described for *Agaricus* (Chen, Stone et al. 2000) with up to 40 % transformation efficiency (regenerating colonies from mycelia on selection medium), but higher than efficiencies of fungal conidia transformation (between 0.03 % and 0.09 % for *Aspergillus awamori* (de Groot, Bundock et al. 1998) or *Fusarium oxysporum* (Mullins, Chen et al. 2001) or many plant protocols (Clough and Bent 1998; Davis, Hall et al. 2009). For the basidiomycete *Hebeloma cylindrosporum,* the number of transformations was mentioned with almost 80 transformants per 100 glass microfiber discs. However, a direct comparison of *Laccaria* and *Hebeloma* transformation efficiencies is not feasible, because fungal macerate and not pieces of fungal colonies were used in the case of *Hebeloma* (Combier, Melayah et al. 2003).
Generally, fungal transformation efficiency is underlying strong variations (mentioned for instance for *Suillus*, *Hebeloma*, and *Paxillus* (Pardo, Hanif et al. 2002)), which was also observed in this thesis. Apart from the large number of possible variations discussed above, yet unknown factors can be supposed that can remarkable modulate transformation efficiency. A nice example are differences in the genotype; which can modulate transformation efficiency by up to two orders of magnitude using the same robust transformation protocol (*Arabidopsis,* (Clough and Bent 1998); *Agaricus bisporus*, (Costa, Thomas et al. 2009)).

4.5 Formation of dikaryons

The formation of dikaryotic mycelia is a prerequisite for development of functional mycorrhiza. Furthermore, dikaryon formation from transgenic monokaryons might enable multiple combinations of mRNA silencing constructs, which is not yet feasible due to the lack of additional selection systems for *Laccaria* transformation.
Due to the fact, that transformation efficiency of the available dikaryotic strain was extremely low, a protocol for the transformation of two compatible monokaryotic *Laccaria* strains was developed in this work. To proof the above mentioned opportunity to target different genes by RNAi-based mRNA suppression in a single fungal strain, dikaryotic mycelia were generated harbouring different combinations of RNAi constructs. The strategy for dikaryon selection out of the monokaryotic background is based on varying resistance of mono- and dikaryotic mycelia to elevated ammonium phosphate concentrations. Thus, the developed strategy was highly efficient, as 50 out of a total of 53 investigated isolates from hyphal fusion approaches revealed their

dikaryotic character by formation of fully developed clamp connections. Fully developed clamp connections are typical only for dikaryons in a number of well known basidiomycotic fungi like *Coprinopsis*, *Hebeloma and Agaricus* (Raper 1966; Kothe 2001; Srivilai and Loutchanwoot 2009; Stajich, Wilke et al. 2010).

Dikaryon formation from compatible monokaryons has been described for a number of basidiomycetes (Petersen and Bermudes 1992; Gharehaghaji, Goltapeh et al. 2007), including *Laccaria* (Bastide, Kropp et al. 1995; Selosse, Costa et al. 1996). All these strategies were based on the isolation of small mycelial patches from the confrontation region followed by microscopic inspection for clamp connections and several rounds of transfer of dikaryon showing mycelial patches to fresh agar plates. The novelty of the strategy that has been developed in this work was the development of a selection strategy for dikarya, making mating much more efficient.

While some combinations of RNAi construct harbouring monokaryons were successfully forming dikaryons other combinations were not (for details see appendix Tab. 8). As the monokaryotic *Laccaria* strains have been shown to be fusion compatible, the reason for the inability of certain transformants to form dikaryons must be based on either a) the integration site of the t-DNA, or b) a lower competitiveness of certain RNAi-harbouring combinations (for instance due to reduced stress tolerance of resulting dikarya under stress conditions as the elevated ammonium).

During hyphal confrontation, a white floccus was formed for certain combinations of monokaryons, which built small concentrated circular areas. Similar structures were described for fruiting initiation of *Coprinopsis cinerea* (Boulianne, Liu et al. 2000; Srivilai and Loutchanwoot 2009) and *Schizophyllum commune* (Ohm, de Jong et al. 2010). However, in *Laccaria* no further development, as described for *Schizophyllum*, was observed even after longer co-culture.

4.6 Strategy for gene silencing in *Laccaria bicolor*

Gene silencing is a common strategy to study fungal physiology, especially when knock out strategies are not feasible due to low rates of homologous recombination (as demonstrated in *Agaricus bisporus* (Costa, Thomas et al. 2009), *Coprinopsis cinerea* (Heneghan, Costa et al. 2007; Costa, Mills et al. 2008), *Schizophyllum commune* (Schuurs, Schaeffer et al. 1997; de Jong, Deelstra et al. 2006), and *Laccaria bicolor* (Kemppainen, Duplessis et al. 2009; Kemppainen and Pardo 2010; Kemppainen and Pardo 2010)). Today, classical antisense strategies (transgene expression of a reverse oriented large fragment of a target gene (Tian and Chen 2001; Li, Zhou et al. 2003)) have been mainly substituted by RNAi-based approaches (expression of short dsRNA-

triggering DNA fragments of up to a few hundred bp (Smith, Singh et al. 2000; Salame, Ziv et al. 2011)).

In this study, RNAi-based silencing of selected genes was performed in *Laccaria bicolor*. Aim was the manipulation of fungal carbohydrate storage (trehalose and glycogen) metabolism. Therefore, two monokaryotic *Laccaria* strains (S238N-H70 and S238N-H82) were transformed with RNAi constructs to manipulate trehalose ((TPS1 (trehalose-6-phosphate synthase 1), TP (trehalose phosphorylase), NT (neutral trehalase)) or glycogen (GS (glycogen synthase)) metabolism. By this approach, either biosynthesis (TPS1 and GS) or degradation (NT and TP) was targeted.

The intent of using two monokaryotic strains in parallel was to enable suppression of different genes by using the same dominant selection system (hygromycin resistance) in a two step procedure. First step was the transformation of monokaryons by the respective constructs and analysis of their phenotype. Second step was the formation of dikaryons by fusion of two compatible monokaryons. Dikaryon formation can be performed with any combination of RNAi construct containing monokaryons; for instance combinations were performed as biosynthesis and degradation of trehalose to be suppressed at the same time. Such an approach is not yet feasible in a single monokaryon, because a second suitable selection system is missing for *Laccaria*. Furthermore, commonly only one nucleus will contain a construct as result of a successful transformation of a dikaryon. In contrast, by fusion of two monokaryons, dikaryons can be generated in which each nucleus does contain its own RNAi cassette, perhaps leading to stronger phenotypes.

The appropriate length of RNAi fragments was often discussed to be essential for strong silencing effects in literature. Constructs with only few complementary bases give rise to only small stem-forming double-stranded interfering RNAs, which are expected to be less suitable to induce gene silencing by DICER-based cleaving the target mRNA (Bernstein, Caudy et al. 2001). In *Neurospora crassa*, strongest gene silencing was observed for stem length between 600 to 900 bp, while fragments shorter than 200 bp were not suitable at all (Goldoni, Azzalin et al. 2004). Accordingly, for all *Laccaria* genes, selected for silencing in this study, a hairpin stem length of close to 500 bp was chosen (if possible due to the given gene-specific exon-intron structure of the gDNA). Furthermore, potency of hairpin RNAs can be significantly increased when a spliceable intron is integrated, serving as connector of the hairpin stem defining fragments. As for instance demonstrated in a comparative *Arabidopsis* research, using hairpin RNA fragments with or without an integral intron, notably the intron variant degreased target protein activity as low as knock out plants (Stoutjesdijk, Singh et al.

2002). Similar results were achieved in *Nicotiana tabacum*. Here, the authors supposed that the intron splicing spliceosome might help to form the double-stranded hairpin by promoting the stem hybridisation. It was further discussed that the splicing per se somehow increased the amount of hairpin RNAs by facilitating the hairpin's passage from the nucleus. Another explanation was a splicing induced creation of a small less nuclease-sensitive loop (Smith, Singh et al. 2000). With respect to this, a maximal repression of 96 % was reached for the *TPS1* gene in *Laccaria*.

The amount of interfering RNA in the cell is directly correlated to the strength of gene suppression (Elbashir, Lendeckel et al. 2001). Therefore, a strong promotor is of prime importance for successful gene silencing. In previous studies, using RNAi-based strategies for gene suppression in *Laccaria*, the *glyceraldehyde-3-phosphate dehydrogenase* promoter (P*gpd*) of *Agaricus* was successfully used (Kemppainen, Duplessis et al. 2009). This promotor is well investigated (Harmsen, Schuren et al. 1992), allowing a strong, constitutive and robust gene expression in a large number of basidiomycetes (*Hypholoma* (Godio, Fouces et al. 2004), *Coprinopsis* (Burns, Gregory et al. 2005), *Pleurotus* (Ding, Liang et al. 2011), *Flammulina* (Kuo, Chou et al. 2004), *Agaricus* (van de Rhee, Graca et al. 1996; Chen, Stone et al. 2000), *Suillus* (Hanif, Pardo et al. 2002), and *Schizophyllum* (Schuren and Wessels 1994; Schuurs, Schaeffer et al. 1997)). Therefore, the *gpd* promotor is driving the expression of all RNAi cassettes constructed in this work.

4.7 Quantification of RNAi-induced gene silencing

Quantification of RNAi-induced gene silencing was determined by using reverse transcriptase-based PCR. By this approach the mRNA content of a given gene is correlated to the RNA content of an appropriate reference gene. Often 18S rRNA is used as reference in *Laccaria* (Lopez, Manner et al. 2007; Lopez, Dietz et al. 2008; Dietz, von Bulow et al. 2011) as well as in other fungi (e.g. *Agaricus bisporus* (Costa, Thomas et al. 2009)). The advantage of using 18S rRNA is that the expression of the respective gene turned out to be stable under most conditions investigated so far (Goidin, Mamessier et al. 2001; Pfaffl 2001; Jarosova and Kundu 2010; Yan, Yuan et al. 2012). The disadvantage is, however, a much higher expression level of 18S rRNA compared to that of target genes (Pfaffl and Hageleit 2001). Similar efficiencies of reverse transcription thus have to be secured by using only low amounts of starting RNA. This strategy reduces the sensitivity of this approach and may cause trouble when analysing lowly expressed target genes (Tricarico, Pinzani et al. 2002; Wong and Medrano 2005). Alternative reference genes (e.g. elongation factor 3 (JGI ID: 293350),

GTPase, or metalloprotease (JGI ID: 245383)) have been suggested for normalisation in *Laccaria* (Deveau, Kohler et al. 2008; Plett, Gibon et al. 2012) but are not yet investigated in much detail. However, PCR reactions have to be rather robust for mRNA quantification. Thus, calibration curves, using template concentrations that differ by several orders of magnitude, are essential to determine PCR quality and the reliable detection range. For all primer pairs amplification slopes between -3.143 (GP) and -3.591 (GS) were obtained, indicating template amplification efficiencies of almost 2 (duplication of template DNA within one cycle). Furthermore, R^2 values between 0.9817 (TP) and 0.9999 (NT) indicates highly stable and reproducible PCR results over a wide range of template concentrations (Meuer, Wittwer et al. 2001; Pfaffl and Hageleit 2001; Pfaffl 2004; Nolan, Hands et al. 2006).

Moreover, the RNAi-target complementary regions of all *Laccaria* genes, targeted for gene silencing, were chosen near to the 5'-end, mainly the first or second exon-intron combinations, while primers for qPCR were designed for the 3'-region, as generally recommended. RT reaction is most accurate at the beginning of strand synthesis (3'-region) while a potential RT maturation reduces its processivity as longer the cDNA is polymerised (especially when using oligo(dT) primers). Furthermore, the longer the mRNA the higher the risk of truncated cDNA fragments, caused by possible mRNA degradation inhibiting full length transcription (Nolan, Hands et al. 2006). However, previous studies using qPCR approaches for quantification of the remaining mRNA contents in RNAi-studies yielded different results, when primer pairs designed for 5'- or 3'-end-located regions of the RNAi target area were used (Shepard, Jacobson et al. 2005; Holmes, Williams et al. 2010). Primers allowing the amplification of the 3'-end flanking region, frequently underestimated the RNAi effect, indicating that the poly-A tail containing part of the targeted mRNA has a higher stability during RNA interference compared to the 5'-region. Accordingly, a risk of uncleaved mRNA residues of the 3'-region might be possible and a possible underestimation of RNAi-meditated repression might occur (based on primer annealing to non-degraded or at least partly degraded target mRNAs) Nevertheless, as discovered especially in the youngest and middle zone, RNAi technique was very potent.

4.7.1.1 Silencing of TPS1 via RNAi

A single fungal colony on an agar plate is a highly organised structure, composed of circular segments where hyphae reached different developmental stages at which dissimilar physiological properties are requested. These properties were taken into

account by analysing gene expression in the different segments of growing fungal colonies (for a summary see Fig. 64).

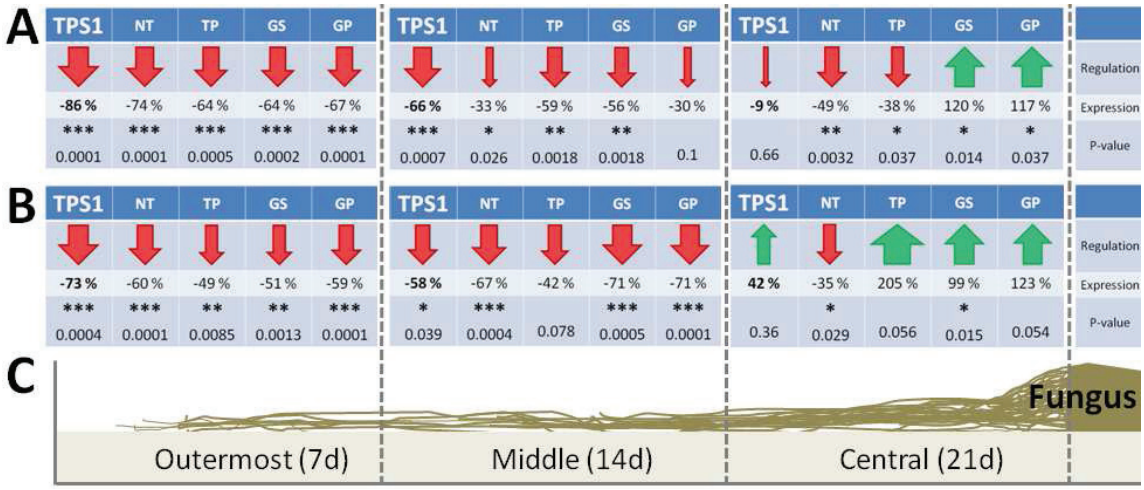


Fig. 64. Changes in gene expression within fungal colonies in TPS1-RNAi transformants calibrated to wild type mycelia
The expression of *TPS1*, *NT*, *GS*, *GP*, and *TP* genes in TPS1-RNAi transformants was calibrated to that of wild type hyphae for colonies dissected into three circular segments (**C**). Shown is the average of all transgenic strains with (**A**) S238N-H70 and (**B**) S238N-H82 background. Arrows highlight the observed changes in gene expression (increase (green), decrease (red)) in those transformants compared to that of wild type hyphae. P-values are indicated as significant (p-value < 0.05 (*)), highly significant (p-value < 0.01 (**)), and extremely significant (p-value < 0.001 (***)).

In transformants with both 238N-H70 and 238N-H82 background, the average of RNAi-based suppression of *TPS1* gene expression was observed in the youngest, outermost area, revealing suppression levels of 86 % (H70 mutants) and 73 % (H82 mutants). In the middle section *TPS1* transcript levels were reduced with 66 % (H70 mutants) and 58 %. Comparable RNAi efficiencies were discovered for human cell lines (Lebbink, Lowe et al. 2011) and also for different fungi (for instance *Coprinopsis cinerea* (Walti, Villalba et al. 2006), *Agaricus bisporus* (Costa, Thomas et al. 2009), and *Neurospora crassa* (Goldoni, Azzalin et al. 2004)), leaving frequently at least 10 % of wild type mRNA levels unaffected. However, a complete different picture was seen for *TPS1* transcript levels detected in the oldest part of the fungal colonies. Here, in mutants of both backgrounds, only TPS1-7010 revealed again a strong suppression of *TPS1*, while five other transformants revealed a weak reduction and four transformants even an increased *TPS1* expression compared to that of the wild type (see Fig. 53). Moreover, a single transformant (TPS1-8208) displayed already in the middle part of the colony no reduction of *TPS1* gene transcription anymore that was discovered in the oldest area (very strong transcriptional induction of almost 130 %), too. The dissimilar

efficiencies of RNAi-based *TPS1* suppression might be explained, in part, by the transcript levels, observed in different parts of the fungal colony, being much higher in the mycelial front and the middle area compared to the oldest, central part. This phenomenon, however, also reflects the importance to take into account the physiological state of the fungus for RNAi suppression capability. Furthermore, in human cells, where similar observations were made, targeting of promoter elements by small mRNA-fragments (generated by the RNAi cascade) was discussed to be the reason for RNAi-induced gene expression (Li, Okino et al. 2006). For *Agaricus*, such an RNAi-induced expression induction was described, too (Costa, Thomas et al. 2009). However, trehalose biosynthesis and break down are tightly controlled in fungi (François, Walther et al. 2012), and metabolisms of trehalose and glycogen are cross-linked at the transcriptional but also posttranscriptional level under most physiological condition (see below) (Parrou, Enjalbert et al. 1999; Guillou, Plourde-Owobi et al. 2004). Therefore, expressions of *TPS1*, *TP*, *NT*, *GP*, and *GS* genes were studied simultaneously for each transformant. All investigated genes revealed high levels of suppression of gene expression in the youngest part of the fungal colony, followed by a less severe reduction in the middle part of the colony. In contrast, gene expression was at, or even above, wild type levels in the oldest part of the fungal colony. Here, *NT* was, however, an exception because transcript levels were still significantly reduced by 49 % in H70 strains and 35 % in H82 strains (see averages in Fig. 64). Taken together, these data clearly indicate intensive co-regulation of all investigated genes at the transcriptional level.

The results obtained for the youngest part of *Laccaria* colonies are in agreement with those of *Saccharomyces cerevisiae*, where *TPS1* deletion resulted in a significant and synchronised decrease in both *GS* and *GP* mRNA levels in growing cells. Furthermore, *TPS1* and *NTH1* expression were co-regulated during heat stress (Winderickx, de Winde et al. 1996; Xu and Tsurugi 2007) and all yeast homologues of the here analysed *Laccaria* genes (*TPS1*, *NTH1*, both *GSY*, and *GPH1*) are expressed in chorus in *Saccharomyces* (Parrou, Enjalbert et al. 1999) as determined regarding all three sectors of both wild types in which the overall expression is simultaneously changed.

Explanations for a concerted expression of genes coding for proteins involved in carbohydrate storage metabolism can be found in cAMP-mediated proteinkinase A (PKA) signalling pathways and the presence of common cis-elements in the promotors of all investigated genes in *Laccaria* (see below).

4.7.1.1.1 cAMP and Hexokinase signalling are involved in regulation of storage carbohydrate content

Transfer of carbohydrate starved yeast cells to a glucose rich medium (Cannon, Gibbs et al. 1986; Cameron, Levin et al. 1988; Portela, Van Dijck et al. 2003) or initialisation of spore germination of *Magnaporthe grisea* (Bourett and Howard 1990; Thines, Weber et al. 2000; Foster, Jenkinson et al. 2003) results in a synchronised trehalose and glycogen degradation. In yeast, glycogen (but not trehalose) content increases slightly during the exponential growth phase. Major trehalose and glycogen accumulation are observed in late logarithmic and early stationary growth phase. Furthermore, massive trehalose formation is induced upon temperature stress (Zahringer, Thevelein et al. 2000) as also common known for basidiomycetes (Tibbett, Sanders et al. 2002; Ngamskulrungroj, Himmelreich et al. 2009; Salmerón-Santiago, Pardo et al. 2011). In late stationary phase, glycogen and, with a short delay, trehalose degradation occurs (Francois and Parrou 2001). Apart from stress response, the physiological meaning of trehalose and glycogen accumulation is somehow different in basidiomycetes (including ectomycorrhizal fungi). Here, trehalose accumulation starts immediately after glucose addition, while glycogen accumulation is retarded (Wallenda 1996; Hoffmann, Wallenda et al. 1997; Felmeth 2012; Samborski 2012). Nevertheless, the basic mechanisms leading to carbohydrate-dependent regulation seem to be similar between asco- and basidiomycetes (Nehls, Gohringer et al. 2010), while mainly fine tuning leads to the observed differences. As regulatory circuits have been investigated best for ascomycetes, the respective regulatory components form the basis for interpretation of results obtained with *Laccaria* (this work).

cAMP levels are a major trigger for regulation of storage carbohydrate metabolism in fungi. Under normal conditions cAMP levels are very low in yeast cells and increase only transiently when environmental changes occur, for example glucose addition to carbon starved cells (Mbonyi, van Aelst et al. 1990; Portela, Van Dijck et al. 2003). Mutations in genes coding for proteins involved in cAMP-dependent protein kinase signalling strongly affected glycogen and trehalose accumulation (Uno, Matsumoto et al. 1983; Matsumoto, Uno et al. 1985; Cannon, Gibbs et al. 1986; Cannon, Pringle et al. 1994). With respect to this, disruption of *TPS1* in yeast did not only prevented cAMP raise after glucose addition (Gonzalez, Stucka et al. 1992), but caused even overall decreased cAMP levels (Noubhani, Bunoust et al. 2009). This is a plausible reason explaining the growth defects of yeast *TPS1* mutants on glucose. However, this abnormal behaviour of cAMP levels could be partly restored by deletion of hexokinase 2 in a *TPS1*-deleted mutant background. Together this clearly indicates the

involvement of TPS1 in regulation of cAMP level and points towards an interaction of TPS1 and hexokinase 2 (Noubhani, Bunoust et al. 2009). This interplay of TPS1 and hexokinase 2 function seemed to be mediated by trehalose-6-phosphate (T6P) (Blazquez, Lagunas et al. 1993), the product of TPS1. T6P levels in cells are regulated by TPS1 (biosynthesis) and TPS2 (degradation, trehalose formation). In agreement with the supposed function of T6P, deletion of TPS2 resulted in extremely increased cAMP levels. Again, deletion of hexokinase 2 in a TPS2 mutant was suitable to restore wild type-like cAMP behaviour (Noubhani, Bunoust et al. 2009). So far, T6P-based regulation of hexokinase function is common knowledge in *Saccharomyces* (Blazquez, Lagunas et al. 1993; Thevelein and Hohmann 1995; Noubhani, Bunoust et al. 2009) and was recently confirmed in *Aspergillus fumigatus* (Puttikamonkul, Willger et al. 2010). Nevertheless, a comparable function of T6P can be supposed for TPS1-RNAi transformants in *Laccaria*, too. A reduction of *TPS1* expression is expected to result in a reduced cellular T6P level. Unfortunately, this effect could not be demonstrated directly, as cellular T6P concentrations were too low for conventional detection methods in *Laccaria* (Samborski 2012). However, in agreement with yeast *TPS1* mutants, revealing a strong glucose-6-phosphate (G6P) accumulation (Blazquez, Lagunas et al. 1993; Thevelein and Hohmann 1995; Noubhani, Bunoust et al. 2009), Samborski (2012) found increased cellular G6P levels in TPS1-RNAi transformants. Furthermore, depletion in ATP due to elevated G6P formation is a main direct reason for growth inhibition of *TPS1* yeast mutants on glucose (Blazquez, Lagunas et al. 1993), indicating a disordered feedback control between hexose uptake and metabolism. Such growth inhibition on glucose, indicated by reduced maximal growth speeds and hyphal densities, was also observed in the S238N-H70 transformants. The absence of growth inhibition in transformants with S238N-H82 background might indicate different threshold levels necessary for T6P function in both *Laccaria* strains.

4.7.1.1.2 PKA-based transcriptional regulation

As consequence of low PKA activity, increased transcript levels of genes coding for proteins involved in biosynthesis but also degradation of trehalose and glycogen are observed (see above), and thus a concerted mechanism to control the expression of these genes is indicated. As carbohydrate storage (but not degradation) is preferred in yeast under these physiological conditions, further posttranscriptional regulation is needed (see below) to drive cellular physiology towards carbohydrate storage (Winderickx, de Winde et al. 1996; Parrou, Teste et al. 1997; Sunnarborg, Miller et al. 2001; Xu and Tsurugi 2006).

The transcription factors MSN2 and MSN4 are substrates of PKA. Both transcription factors together allow the expression of nearly 200 genes necessary for cellular response to a changing environment, including heat and cold shock, osmotic shock, oxidative stress, low pH, and glucose starvation (Gorner, Durchschlag et al. 1998; Thevelein and de Winde 1999). Phosphorylation of MSN2 and MSN4 by PKA avoids the nuclear localisation of these proteins (Varela, Praekelt et al. 1995; Martinez-Pastor, Marchler et al. 1996; Gorner, Durchschlag et al. 1998) leading to a reduced transcriptional activity in the presence of active PKA (see below). Those two factors regulate gene expression by binding to so-called STRE elements (5'-CCCCT-3') (Martinez-Pastor, Marchler et al. 1996; Gorner, Durchschlag et al. 1998; Gasch, Spellman et al. 2000; Causton, Ren et al. 2001; Gorner, Durchschlag et al. 2002; Kandror, Bretschneider et al. 2004). STRE elements are routinely found in the promoters of yeast genes, coding for proteins involved in storage carbohydrate biosynthesis and degradation, explaining why the entire metabolisms of trehalose and glycogen are activated at the transcriptional level in a PKA-dependent manner upon carbon starvation. The numbers of STRE elements, found in the respective promotors, directly correspond to the expression strength of many genes (Varela, Praekelt et al. 1995; Gorner, Durchschlag et al. 1998). This could be further confirmed by artificial promoters and STRE element deletion studies (Kobayashi and McEntee 1990; Marchler, Schuller et al. 1993). However, such a strict correlation is not always observed. In heat-stressed yeast cells, *GSY1* (two STRE elements) was much higher expressed than *TPS1* (six STRE elements), indicating the interplay of MSN2 and MSN4 with other regulatory factors for regulation of transcription of certain STRE element containing genes.

Homologues of MSN-like transcription factors were identified in the *Laccaria* genome (this work). Furthermore, putative STRE elements were identified in the promotor regions of *Laccaria* genes coding for proteins involved in trehalose (*TPS1*, *NT*, and *TP*) and glycogen (*GS* and *GP*) metabolism (this work). Two STRE elements could be detected in the promotor of *NT*, three in those of *GS* and *TP*, five in the *TPS1* gene, and eight in the promotor of *GP* (this work).

The presence of MSN-like transcription factors, together with the observed putative STRE elements, makes a PKA-based transcriptional regulation of *TPS1*, *NT*, *TP*, *GS*, and *GP* thus rather likely. In agreement with this hypothesis, a coordinated repression of *TPS1*, *NT*, *TP*, *GS*, and *GP* expression was detected in *TPS1*-silenced mutants. Suppression of gene expression was, however, restricted to younger parts (outermost and middle sections) of a growing fungal colony. A major difference between younger

and older parts of *Laccaria* colonies on agar plates is the availability of external glucose, which will be best at the hyphal front. In agreement with data from *Amanita* where a temporary rise in cAMP level was followed by trehalose accumulation (that was highest when cAMP started to decrease), transcript levels for *TPS1* were highest in the mycelial front in *Laccaria*, again indicating a glucose-based regulation of gene expression. Also in agreement with a PKA-based coordination of gene expression, highest transcript levels for all investigated genes, coding for proteins involved in storage carbohydrate metabolism, was observed at the hyphal front in colonies with S238N-H70 background. *Laccaria* colonies with a S238N-H82 background, however, revealed similar high transcript levels of all investigated genes at the hyphal front as well as the middle section of growing colonies. As speculated above for the impact of TPS1-RNAi effect on maximal fungal growth speed, the observed differences in gene expression pattern in colonies with S238N-H70 and S238N-H82 background might be explained by different threshold levels for PKA-based regulation.

4.7.1.1.3 PKA-based post-transcriptional regulation of storage carbohydrate metabolism

In yeast, the expression of genes involved in storage carbohydrate metabolism (degradation and biosynthesis) is rather low due to PKA activity when carbon starved cells are transferred into a good carbohydrate source (see above). It must therefore be concluded that gene expression and protein function must be somehow uncoupled to explain the preferential degradation of storage carbohydrates under these conditions in yeast and other ascomycetes.

Apart from suppression of gene expression (see above) elevated PKA activity leads to protein activation (by direct phosphorylation) of NTH1 (neutral trehalase) in yeast (Mbonyi, van Aelst et al. 1990; Gonzalez, Stucka et al. 1992; Arguelles, Carrillo et al. 1993; Souza, De Mesquita et al. 2002). At the same time TPS1 activity is reduced by almost 50 % (Panek, de Araujo et al. 1987; Vandercammen, Francois et al. 1989; Arguelles, Carrillo et al. 1993; Reinders, Burckert et al. 1998) when present as complex with other regulatory subunits (see below) potentially by TOR kinase (Pedruzzi, Dubouloz et al. 2003; Loewith 2011). In *Saccharomyces* (Vandercammen, Francois et al. 1989; Panek, Araujo et al. 1990; Londesborough and Vuorio 1993) and *Magnaporthe grisea* (Wilson, Jenkinson et al. 2007), TPS1 activity seemed to be, however, more affected by free phosphate than by phosphorylation. TPS1 activity (free protein) is increased by elevated phosphate concentrations (Bell, Sun et al. 1998) while

its activity is inhibited under these conditions when TPS1 is forming a complex with two regulatory subunits, TSL1 and TPS3.

Similar to the situation in trehalose metabolism, key proteins of glycogen metabolism are also regulated by phosphorylation. Phosphorylation of GSY1 (glycogen biosynthesis) leads to protein inhibition, while phosphorylation of GPH1 (glycogen degradation) result in activation of protein activity in yeast (Francois and Parrou 2001; Wilson, Wang et al. 2005; François, Walther et al. 2012) as response to elevated cAMP levels. However, no direct evidence for a PKA-dependent phosphorylation of both are known so far (François, Walther et al. 2012), but it was shown that PKA phosphorylates GPH1 *in vitro* (Lin, Hwang et al. 1995). A similar mechanism was described for the basidiomycete *Coprinopsis*, in which the GP is activated by phosphorylation as a consequence of increased cAMP levels (Uno and Ishikawa 1976) while the GS is inactivated (Kuhad, Rosin et al. 1987), indicating well conserved regulatory networks.

Taken together, independent on gene expression, high cAMP and PKA level-based phosphorylation of proteins, involved in biosynthesis (inactivation) and degradation (activation) of storage carbohydrates, leads to trehalose and glycogen degradation in yeast and other organisms.

Moreover, strong TPS1 and NTH1 gene expression was, however, observed during heat stress, while only minor NTH1 but high TPS1 protein activity was found, leading to trehalose accumulation as consequence. The noticed low PKA activity could explain both, high transcript levels and trehalose and glycogen accumulation by a low phosphorylation status of key transcription factors and proteins involved in trehalose and glycogen metabolism at these conditions (Francois, Eraso et al. 1987; Zahringer, Holzer et al. 1998; Zahringer, Thevelein et al. 2000; Francois and Parrou 2001).

Similar to yeast, cAMP levels rise upon transfer of carbon starved hyphae into glucose rich medium in *Amanita* (Hoffmann, Wallenda et al. 1997). However, dissimilar to yeast, genes involved in storage carbohydrate metabolism are synchronously induced in *Laccaria* and trehalose accumulation (instead of break down as in yeast) occurs as consequence (this work). However, synchronisation of expression of genes involved in storage carbohydrate metabolism, the presence of MSN-like transcription factors in the *Laccaria* genome (this work) and STRE elements in the promotor region of respective *Laccaria* genes (this work) make a PKA-based regulation of storage carbohydrate metabolism in *Laccaria* rather likely. The only difference to yeast, that has to be postulated for *Laccaria,* would be cAMP-based inactivation of PKA. A cAMP-based reduction of PKA activity in *Laccaria* hyphae shifted from carbohydrate poor to rich medium would result in:

a) the observed strong expression of genes involved in storage carbohydrate metabolism (this work) due to a low phosphorylation status of MSN-like transcription factors, and

b) a low phosphorylation status of NT and thus trehalose accumulation as observed in the mycelial front of wild type *Laccaria* colonies (Samborski 2012). A correlation between low NT activity and a low protein phosphorylation status and vice versa has already been shown for *Amanita* (Wisser 2001).

How do results, obtained with TPS1-RNAi constructs, fit into this picture? The concerted reduction in transcript levels of all genes involved in storage carbohydrate metabolism, in most of the TPS1 RNAi transformants, indicates a regulatory cause. cAMP levels, TPS1 protein activity, trehalose-6-phosphate decrease, and glucose-6-phosphate increase are discussed in yeast as potential reasons for such a broad impact. In TPS1-RNAi transformants of *Laccaria,* glucose-6-phosphate levels were clearly increased (up to a factor of two). In yeast and other ascomycetes, this increase would have had, however, no severe impact (Smallbone, Malys et al. 2011). Trehalose-6-phosphate levels were below the detection limit and TPS1 activity as well as cAMP levels have not been determined yet in TPS1-RNAi transformants. The cause for the reduced transcript levels of genes involved in storage carbohydrate metabolism remains therefore obscure. Nevertheless, if transcription is regulated as supposed above, increased PKA levels must be expected. This increased activity would result in increased NT and (perhaps) reduced TPS1 activities due to a higher phosphorylation state of the proteins. Nevertheless, trehalose content did not decrease below wild type level in the hyphal front of *Laccaria* TPS1-RNAi transformants. Therefore, either PKA activity does not increase in TPS1-RNAi transformants or increase in PKA activity must be compensated by elevated NT dephosphorylation (by phosphatases). Another potential NT regulation might by a direct inhibitor-protein, as known in yeast (de Mesquita, Paschoalin et al. 1997). The decrease in glycogen content, as observed in certain TPS1-RNAi transformants (Felmeth 2012), would be, however, in agreement with a supposed increase in PKA activity in these transformants. As mentioned above, elevated cAMP-levels, and thus most potentially increased PKA activity, results in phosphorylation of GS (reduced glycogen biosynthesis as consequence) and GP (leading to increased glycogen degradation), which results in overall glycogen degradation (as demonstrated for yeast and *Coprinopsis*). Therefore, phosphatase regulated reduction in the phosphorylation status of NT or a direct regulatory protein might be the best explanations for the observed phenotypes of TPS1-RNAi transformants.

But why do TPS1-RNAi transformants with S238N-H70 or S238N-H82 background differ in their growth behaviour? One explanation might be a differential expression of mating type genes, which is indicated by the formation of pseudo clamps by S238N-H70 but not S238N-H82, a process known to be under mating type control. In *Ustilago maydis* one regulatory subunits (115 kDa; TPS3 or TSL1) of the TPS complex has been shown to be expressed in a pheromone-dependent manner (Zarnack 2006; Zarnack, Maurer et al. 2006) and pheromones are known to integrate nutrient availability into the fungal mating type background, mostly frequently via PKA and MAPK (mitogen activated protein kinase) signalling. The authors supposed that the TPS-complex is important to sense the cellular carbohydrate status which is than reflected into cellular metabolism by PKA-signalling (Zarnack 2006; Zarnack, Maurer et al. 2006).

4.7.1.2 Trehalose function in temperature stress in *L. bicolor*

Apart from its function in carbohydrate storage, trehalose has been shown to act as a metabolite, which highly accumulates in response to stress in fungi (Attfield 1987; Arguelles 1997; Gancedo and Flores 2004; Avonce, Mendoza-Vargas et al. 2006; Al-Bader, Vanier et al. 2010). With regard to temperature, trehalose accumulation was observed at low (Niederer, Pankow et al. 1992; Tibbett, Sanders et al. 2002) but also high temperatures (Van Aelst, Hohmann et al. 1993; Blazquez and Gancedo 1994; Wolschek and Kubicek 1997; Shinohara, Correa et al. 2002; Petzold, Himmelreich et al. 2006). Under these conditions, TPS1 as well as TPS2 transcript levels were highly elevated (Elliott, Haltiwanger et al. 1996; Ocon, Hampp et al. 2007). TOR and PKA signalling induced MSN4- and to a lesser extend MSN2-based regulation is responsible for this increase in transcript levels in yeast (Gasch, Spellman et al. 2000).

Apart from a general slower hyphal growth, cold temperature (12 °C) reduced growth suppression of TPS1-RNAi containing *Laccaria* transformants. However, exposure to heat stress (26 °C and above) clearly revealed lower resistance of transformants with S238N-H70 background compared to the respective wild type. The transformant with the lowest TPS1 expression (TPS1-7010) completely died at 28 °C, while other transformants with a less severe reduction in their TPS1 level displayed sectoral growth pattern but survived. Even when similar effects on the suppression of TPS1 expression were observed for both *Laccaria* strains, transformants with a S238N-H82 background did not obviously differ from the respective wild type with regard to their sensitivity to elevated temperature. However, colonies of those transformants that

revealed strong TPS1 silencing effects (TPS1-8206, TPS1-8207) seemed to be under physical tension, a phenomenon that was never observed in wild type colonies.
One possible reason for the lower heat sensitivity of transformants with S238N-H82 background compared to those with a S238N-H70 background might be the fact that they have a higher trehalose content (under non-stress conditions). However, data on the trehalose content under elevated temperature are necessary to proof this hypothesis.

4.8 Conclusion TPS1 in *Laccaria bicolor*

In *Laccaria* the PKA is an ideal candidate causing the significant reduced expressions of all four unsilenced genes in chorus with the TPS1-silenced expression. This becomes plausible regarding the MSN factor mediated control of STRE element encoding genes. In yeast, PKA phosphorylates the MSN factors which are then no longer able to be translocated into the nucleus to induce gene expression. Such a mechanism in *Laccaria* is strongly promoted by the fact that the GP is mostly strongest transcribed in both wild types, which can be correlated with its high number of promotor STRE elements. Furthermore, the expressional activity in the H70 and H82 is strongest at the outermost parts of the colony, the area of potentially highest glucose uptake, and diminishes regarding the inner parts of the colony. This strong expression at the area of highest glucose availability indicates that the expression of all storage carbohydrate-dependent genes is correlates with the glucose supply. This implies that the PKA must be reduced because the putative MSN-like proteins of *Laccaria* seemed to induce the gene transcription in the nucleus.
Such a function of a PKA is promoted by an *Ustilago maydis* research from 2006. In this study, a distinct connection of PKA phosphorylation dependent expression and TPS-complex were discovered (Zarnack 2006; Zarnack, Maurer et al. 2006). They identified that the 115 kDa subunit of the TPS-complex (accession: um02390) was significantly transcribed as response to pheromones indicating sufficient nutrients and reduced after PKA-mediated inhibition of transcription factor PRF1 (pheromone response factor) (Kaffarnik, Muller et al. 2003; Feldbrugge, Kamper et al. 2004; Zarnack 2006). Pheromones are common known in *Ustilago* and other basidiomycetes to integrate nutrient availability into the fungal mating and nutrition physiology, mostly via PKA and MAPK signalling. The authors supposed that the TPS-complex is somehow important for the overall main physiology in response to high nutrients, induced by pheromones and reduced by PKA-signalling through cAMP (Zarnack 2006; Zarnack, Maurer et al. 2006). In addition, this PRF1 performed very similar to the

Saccharomyces MSN factors, also via cAMP induced PKA activity. In another very early *Ustilago* research, the specificity of trehalose synthesis as response to uptaken glucose was demonstrated. Trehalose was only synthesised when glucose was available in the artificial cultures. In case of other carbohydrates like fructose mainly mannitol and erythritol were accumulated. Both last mentioned carbohydrates were further excreted into the growth medium by the fungus, but not trehalose (Gaunt and Manners 1973). Furthermore, in the basidiomycotic yeast *Cryptococcus,* the TPS1 and other TPS-complex genes were significantly transcribed during sufficient glucose supply (Steen, Zuyderduyn et al. 2003; Perfect 2005; Petzold, Himmelreich et al. 2006; Ngamskulrungroj, Himmelreich et al. 2009) matching to the *Ustilago* research and the *Laccaria*-specific data of this thesis. Taking together, on the one hand basidiomycetes seemed to induce a yet unknown signal when exposed to sufficient nutrients leading to both transcription of storage dependent genes and activation of carbon storage generating proteins, for instance the TPS-complex as well as the GS. On the other hand, they reduce the transcription as well as the syntheses of storage compounds or even induce their degradation, via PKA mediated phosphorylation inhibition of MSN-like proteins as well as activation of NT and GP enzyme activity, when insufficient nutrition occur.

However, all results achieved in research, regarding TPS1-silenced *Laccaria,* indicate a somehow high active PKA due to TPS1-silencing, which is absolute contradictory to the yeast TPS1-deletion mutants. For instance, in yeast the TPS1-deletion leaded to diminished cAMP levels and thus to decreased PKA activity causing glycogen hyperaccumulation (possibly due to a reduced GP and induced GS activity). In the TPS1-silenced *Laccaria* strains of both backgrounds, all selected carbohydrate dependent genes are strongly downregulated in the areas of highest glucose nutrition, potentially caused by yeast like MSN factor mediated regulation. Thus, the putative MSN-like factors of *Laccaria* might also be inhibited in their nuclear migration via PKA phosphorylation, promoted by the fact that a common (yeast-like) mechanism seemed to be active with STRE element-/PKA-controlled transcription of all those five genes. In conclusion, the repression of all genes in *Laccaria* might be caused by a high active PKA. This indicated high active PKA is further promoted by the fact that, at least, the H70 TPS1-silenced derivates displayed reductions in trehalose (only TPS1-7010 (Samborski 2012)) as well as glycogen contents. With respect to this, it is plausible the high active PKA has further activated degradative NT and GP as well as inactivated synthetic TPS1 and GS via phosphorylation as common known in yeast. The alleviated effect in trehalose degradation is potentially caused by the basidiomycotic TP, which

might counteract the trehalose depletion. However, the reduced transcription together with the reduced levels of both carbon storages potentially denotes a kind of glucose resistance, because during all assays glucose was abundant and, at least in wild types, transcription of the five genes, as well trehalose and glycogen accumulation were very potent (Felmeth 2012; Samborski 2012). Thus, in wild type *Laccaria* the PKA seemed to be somehow inactivated in case of continuous glucose rich conditions and further activated when depletion of glucose occurs to induce breakdown of internal storages and to repress transcription of the synthetic enzymes and deactivated in proper conditions. Such a mechanism is promoted by studies regarding starved *Coprinopsis* cell-cultures, in which increased cAMP levels were observed (Uno and Ishikawa 1974).

5 Outlook

Even when cellular responses of transformation with TPS1-RNAi cassettes are obvious, copy numbers and integration sites of T-DNA within genomic DNA have still to be determined for selected *Laccaria* transformants. This could help to understand the differences in RNAi efficiency between independent fungal transformants.

While transcript levels seem to have a strong impact on TPS1 protein activity in other fungi, central enzymes for trehalose degradation and glycogen metabolism are regulated mainly at the posttranslational level by their phosphorylation status. The correlation of transcript level and protein activity is, however, yet unknown for TPS1 in *Laccaria*. It has to be determined for interpretation of the observed physiological responses of RNAi-based TPS1 suppression in future.

A striking feature of those transformants, that revealed high RNAi-based TPS1 suppression, is that glycogen but not trehalose content was obviously reduced. Based on our current knowledge, this behaviour can be explained best if cAMP dependent protein kinase A-based co-regulation of protein activity in trehalose and glycogen metabolism is counteracted by increased dephosphorylation of NT. A comparison of the phosphorylation status of NT in wild type and transgenic mycelia would thus be helpful.

Frequently mannitol and other polyols are found to be alternative storage and stress response carbohydrates in other fungi. Induced reduction of TPS1 transcription leads to reduced thermotolerance of monokaryons in S238-H70 background; *Laccaria* is known to be capable to synthesise mannitol (a potential stress competitor), thus the soluble carbohydrate content should be compared between wild type and transgenic strains under heat stress conditions to elucidate both the role of trehalose and mannitol.

As fusion compatible *Laccaria* monokaryons were simultaneously transformed with all RNAi constructs (TPS1, NT, TP, GS, and GP) developed in this thesis, the impact of concerted RNAi suppression on fungal physiology can be analysed. Dikaryons with certain combinations of TPS1- and NT-silenced monokaryons are already available and could thus be tested first as proof of function.

In yeast, a close connection of trehalose synthesis and hexose transport was described. Hence, STRE (stress responsive) elements were observed within the promotors of TPS1 and certain hexose transporter genes, allowing synchronous transcription of the respective genes. Such transcriptional co-regulation was also already observed for certain hexose importers and TPS1 in *Laccaria*. The inspection of

hexose transporter promotors for STRE elements would thus be consequent to understand regulatory connections between glucose import and canalization into storage carbohydrate metabolism.

Nitrogen assimilation is dependent on carbohydrates and a direct link between trehalose metabolism and nitrogen availability has been demonstrated for yeast and *Magnaporthe*. Furthermore, the addition of ammonium to nitrogen but not carbohydrate starved mycelia resulted in strong trehalose and glycogen degradation in *Amanita*. To investigate the impact of carbohydrate storage metabolism on nitrogen assimilation in *Laccaria*, nitrate application to nitrogen starved mycelia of wild type and TPS1-RNAi transformants should be performed. Afterwards, determination of storage carbohydrate content would give first impressions on the physiological link between nitrogen and carbohydrate metabolism in this fungus.

6 List of figures

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8 Literature

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9 Abbreviations

µ	Micro
35S-term	Terminator of transcription
A	Adenine
aa	Amino acid
aadA (Ω)	Spectinomycin resistance gene
aadA_KanR	Kanamycin resistance gene
Abi	*Agaricus bisporus*
AG	Aktiengesellschaft (German)
AGT1	*Saccharomyces* trehalose transporter
Ani	*Aspergillus niger*
Ath	*Arabidopsis thaliana*
AM	Arbuscular mycorrhiza
AMP	Adenosine monophosphate
Ap^R	Ampicillin resistance gene
AS	Acetosyringone
AT	Acid trehalase
ATG	Start of transcription
ATH1	Acid trehalase 1
ATP	Adenosine triphosphate
A. bisporus	*Agaricus bisporus*
A. muscaria	*Amanita muscaria*
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
Ban	*Belgica antarctica*
bar	BASTA resistance gene from *Streptomyces*
BASTA	Glyphosinate ammonium
bla_ApR	ß-lactamase (ampicillin resistance)
BLAST	Basic Local Alignment Search Tool
bom_pBR322	Bom (basis of mobilisation) site from plasmid pBR332
bp	Base pair
BROWSE	JGI website search tool genome browsing
B.V.	Besloten vennootschap (Dutch)
C	Cytosine
CA	California
cAMP	Cyclic adenosine monophosphate
CaMV	Cauliflower mosaic virus
Cci	*Coprinopsis cinerea*
Ccp-9	TP of *Neurospora*
CDC42	Cell division cycle 42 protein
cDNA	Copy DNA
CNRS	Centre national de la recherche scientifique (French)
Co	Common
Co. KG	Compagnie Kommanditgesellschaft
CP	Crossing point
CTAB	Cetyltrimethylammonium bromide
CW	Cell wall
Cys	Cystein
C.	*Coprinopsis*
d	Day
D	Dexter
DAPI	4'-6-Diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_2O	Double distilled water
ddNTP	Dideoxyribonucleotide
DEPC	Diethyl pyrocarbonate
DICER	Adopted from: to dice
dGTP	Deoxyguanosine triphosphate
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide
DOE	Department of energy
Dr. rer. nat.	Doctor rerum naturalium
dsRNA	Double stranded RNA
dTTP	Deoxythymidine triphosphate
E	Efficiency
ECM	Ectomycorrhiza
Eco	*Escherichia coli*
eco47IR	Lethality inducing gene
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced GFP
Eni	*Emericella nidulans*
EST	Expressed Sequence Tags
et al.	*Et alii* (and other)
Ex	Exon sequence
Exin	Exon fragment with surplus intron sequence
Expr.	Expression
E. coli	*Escherichia coli*
e.g.	*Example gratia* (for example)
Fig.	Figure
for	Forward
f1_IG	Intergenic region of phage f1
G	Guanine
g	Acceleration of gravity (≈ 9.81 m/s^2)
GDB1	Glycogen debranching enzyme
gDNA	Genomic DNA
GFP	Green fluorescent protein
Glc	Glucose
GLC3	Glycogen debranching enzyme
GLG	Glycogenin
GLK	Glucokinase
GmbH	Gesellschaft mit begrenzter Haftung (German)
GP	Glycogen phosphorylase
gpd	Glyceraldehyde-3-phosphate dehydrogenase
GPD1	Glycerol-3-phosphate dehydrogenase
GPH	Glycogen phosphorylase of yeast
GS	Glycogen synthase
GSY	Glycogen synthase of yeast
GTP	Guanosine triphosphate
G-protein	GTP-binding protein
G6P	Glycose-6-phosphate
G6PDH	Glycose-6-phosphate dehydrogenase
G.	*Glomus*
HD	Homeodomain transcription factor
hph	Hygromycin resistance gene

hph_HygroR	Hygromycin resistance gene
HXK	Hexokinase
Hygro	Hygromycin
H_2Odd	Water double distilled
ID	Identity
Inc.	Incorporation
INRA	Institut national de la recherche agronomique (French)
IR	Inverted repeat
KEGG	JGI website search tool for metabolic pathways
JGI	Joint Genome Institute
K	Kilo
Kb	Kilo base
KF	Kommanditgesellschaft (German)
KGaA	Kommanditgesellschaft auf Aktien (German)
KM	Substrate concentration at which the reaction rate is half of Vmax
KOG	JGI website search tool for classified proteins
L	Litre
lac	β-galactosidase
lacZ	N-terminal fragment of ß-galactosidase
Lb	*Laccaria bicolor*
Lbi	*Laccaria bicolor*
LB	Lysogeny broth
LB	Left border
log	Logarithmic
Ltd.	Limited
L.	*Laccaria*
m	Milli
M	Molar
MAPK	Mitogen-activated protein kinase
mbar	Millibar
MCS	Multiple cloning site
MD	Maryland
MES	2-(N-morpholino)ethanesulfonic acid
Mg	Magnesium
MI	Michigan
min	Minute
MMN	Modified Melin Norkrans medium
MO	Missouri
MOPS	3-(N-morpholino)propanesulfonic acid
MPDH	Mannitol-1-phosphate 5-dehydrogenase
mRNA	messenger RNA
MSN	Transcription activator of *Saccharomyces*
MtDH	Mannitol dehydrogenase
M.	*Magnaporthe*
n	nano
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NaAc	Sodium acetate
NCBI	National Centre for Biotechnology Information
Ncr	*Neurospora crassa*
NEB	New England Biolabs
NMR	Nitrogen metabolite repressor
No.	Number
NR	Nitrate reductase
NT	Neutral trehalase
NTH	Neutral trehalase of yeast
NY	New York
OD	Optical density
ORLA	TPS2 of *Emericella*
ori_pBR322	Origin of replication from plasmid pBR322
OTSA	Trehalose-6-phosphate synthase of bacteria
P(i)	Inorganic phosphate
p	Plasmid
P	Promotor
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pg	Pico gram
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity
PKA	cAMP dependent protein kinase A
PlacUV5	Modified P_{lac} promotor for eco47IR expression
polyA	Polyadenylation signal
Pos	*Pleurotus ostreatus*
PP(i)	Inorganic diphosphate
Ppu	*Pleurotus pulmonaris*
PPP	Pentose phosphate pathway
Prof.	Professor
Psa	*Pleurotus sajor-caju*
Pt	*Populus trichocarpa*
PTGS	Posttranscriptionally gene silencing
P. tinctorius	*Pisolithus tinctorius*
P.trichocarpa	*Populus trichocarpa*
p.A.	*Pro Analysi*
qPCR	Quantitative real-time PCR
RB	Right border
rep_pMB1	replicon from pMBI plasmid
rep_pVS1	Replicon of plasmid pVS1
rev	reveres
rDNase	Recombinant Desoxyribonuclease
RGT2	Glucose sensor of *Saccharomyces*
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA Interference
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Rounds per minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
S	Sense
Sc	*Saccharomyces cerevisiae*
Sce	*Saccharomyces cerevisiae*
Sco	*Schizophyllum commune*
SDS	Sodium dodecyl sulphate
SGA1	Amylo-glucosidase of *Saccharomyces*
siRNA	Small interfering RNA
Sm^R	Streptomycin resistance gene
SNF3	Glucose sensor of *Saccharomyces*
Spc^R	Spectinomycine resistance gene
SSP	Small secreted protein
sta_pVS1	Stability region of plasmid pVS1
Std.	Standard deviation
STE3	G-protein-coupled pheromone receptor
STRE	Stress responsive element

SUC2	*Saccharomyces* invertase
SWEET	High capacity sugar transport protein
s. a/n. v	Société anonyme (French)/Naamloze vennootschap (Dutch)
S. cerevisiae	*Saccharomyces cerevisiae*
T	Thymine
T4	Bacteriophage T4
T7	Bacteriophage T7
Tab.	Table
t-DNA	Transfer DNA
TAE	Tris-Acetate-EDTA (Buffer)
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TF	Transcription factor
TM	Registered trade mark
TOR	Target of rapamycin
TP	Trehalose phosphorylase
TPP	Trehalose-6-phosphate phosphatase
TPS	Trehalose-6-phosphate synthase
TPSA	Trehalose-6-phosphate synthase of *Aspergillus*
TPSB	Trehalose-6-phosphate synthase of *Aspergillus*
TPS2	Trehalose-6-phosphate phosphatase
TRE	Trehalase
TREA	Trehalase of *Escherichia*
TREB	Trehalose transporter of *Escherichia*
TREC	Trehalose-6-phosphate hydrolase of *Escherichia*
TREF	Trehalase of *Escherichia*
TRE1	Trehalose transporter of *Belgica*
TRE1	Neutral trehalase of *Botrytis*
TRE2	Neutral trehalase of *Neurospora*
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TS	Trehalose synthase
TSB	Transformation and Storage Buffer
TSL1	Trehalose-6-phosphate synthase like 1
T6P	Trehalose-6-phosphate
U	Unit
UDP	Uridine diphosphate
Uma	*Ustilago maydis*
USA	United States of America
UTP	Uridine triphosphate
UV	Ultraviolet
U. maydis	*Ustilago maydis*
U.S.	United States
V	Speed
vir	Virulence gene
V_{MAX}	Maximal speed of a reaction
Vol.	Volume
vol.	Volume
v/v	Volume per volume
WA	Washington
WI	Wisconsin
w/v	Weight per volume
YEB	Yeast extract broth

10 Danksagung

An dieser Stelle meiner Dissertation möchte ich allen danken, die zu dem Gelingen dieser Arbeit beigetragen haben. Zuerst der **Universität Bremen** für die Finanzierung meiner Stelle und meiner Forschungsarbeit. Dann möchte ich natürlich meiner **Prüfungskommission** danken, Herrn Prof. Dr. Uwe Nehls und Frau Prof. Dr. Barbara Reinhold-Hurek für das Erstellen der Gutachten, sowie Frau Prof. Dr. Friederike Koenig, Frau Dr. Andrea Krause, Herrn Dimitri Neb und Herrn Christian Arend als weiteren Mitglieder meiner Prüfungskommission.

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Des Weiteren möchte ich an dieser Stelle meiner Freundin **Nina** (und den drei Schweinen) danken, für ihre bedingungslose Hilfe und Unterstützung in jeder Phase meiner Doktorarbeit. Ohne Sie würde diese Dissertation definitiv nicht vorliegen.

Ich hoffe, ich habe niemanden vergessen und wenn doch, dann wissen die Betroffenen bestimmt, dass ich auch ihnen für ihre Unterstützung dankbar bin.

11 Erklärung

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel „Trehalose in the bicoloured deceiver (*Laccaria bicolor*)“ selbstständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Bremen, 5. April 2013

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Sebastian Wittulsky

12 Curriculum vitae

Personal details:

Family name:	Wittulsky
First name:	Sebastian
Date of birth:	03.03.1983
Place of birth:	Walsrode

Education:

Since 10/2009	Doctorate at the University of Bremen, Faculty 2 (Biology/Chemistry), Division: Botany (Prof. Dr. Uwe Nehls), Germany Title of thesis: Trehalose in the bicoloured deceiver *(Laccaria bicolor)*
9/2009	Diploma in Biology at the Department of Physiological Ecology of Plants (Prof. Dr. Rüdiger Hampp), Faculty of Biology, University of Tübingen Germany Title of thesis: Funktionelle Charakterisierung von Zuckertransportern der Pappel – *Functional analysis of sugar porters in poplar*
10/2004 – 9/2009	Studies of Biology at the Eberhard Karls University of Tübingen, Germany
08/2003 – 07/2004	Berufsoberschule Technik an der Berufsbildenden Schule 3 der Region Hannover, Hannover, Germany
08/2002 – 06/2003	Fachoberschule Agrarwirtschaft an der Justus-von-Liebig Schule, Hannover, Germany

Professional experience:

Since 09/2009	Scientific co-worker at the University of Bremen, Faculty 2 (Biology/Chemistry), Division: Botany (Prof. Dr. Uwe Nehls), Germany
04/2009 – 09/2009	Student research assistant at the Department of Physiological Ecology of Plants (Prof. Dr. Rüdiger Hampp), University of Tübingen, Germany
06/2008 – 02/2009 and 02/2007 – 04/2007	Student research assistant at the Centre for Plant Molecular Biology (ZMBP), Department of Plant Biochemistry (Prof. Dr. Georg Felix), University of Tübingen, Germany
08/2007 – 09/2007	Industrial placement at the sugar beet breeder Diekmann GmbH & Co. KG in Nienstädt, Germany
08/1999 – 06/2002	Apprenticeship as gardener, Specialisation: Nursery, Bruns Pflanzen-Export GmbH & CO. KG, Bad Zwischenahn, Germany

13 Appendix

13.1 Technical equipment

Tab. 14. Technical laboratory equipment

Spectrophotometer: Nano Drop™ DN1000 (PEQLAB Biotechnologie GmbH, Erlangen, Germany) Cell density meter: Biochrom WPA CO8000 (Biochrom Ltd., Cambridge, United Kingdom)
Thermoshaker: Eppendorf Thermomixer 5436 (Eppendorf AG, Hamburg, Germany)
Centrifuges: Eppendorf Centrifuge 5804R; rotor A-2-DWP (2 x microtitre plates), F-34-6-38 (6 x max. 84 mL or 15/50 mL Falcon® tubes with corresponding adapter) (Eppendorf AG, Hamburg, Germany) Eppendorf Centrifuge 5417 R; rotor F 45-30-11 (30 x 1.5/2 mL micro test tubes) (Eppendorf AG, Hamburg, Germany) Heraeus Biofuge Fresco; rotor 3325 (24 x 1,5/2 mL micro test tubes) (Fisher Scientific GmbH. Schwerte, Germany)
PCR-Thermocycler: peqlab primus 25 advanced (PEQLAB Biotechnologie GmbH, Erlangen, Germany) Biometra TGradient Thermoblock (Biometra GmbH, Göttingen, Germany) Biometra PC Personal Cycler (Biometra GmbH, Göttingen, Germany) Bio-rad MyiQTM Real-Time PCR machine (Bio-Rad Laboratories GmbH, Munich, Germany) Roche LightCycler® 480II Real-Time PCR System (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany)
Incubators: Certomat®H, type 886342/3 (B. Braun Biotech International GmbH, Melsungen, Germany) Refrigerator FKS 5000, type 200071, Liminincube II (Analis s.a/n.v, Suarlée, Belgium) In case of liquid cultivation of fungi or bacteria, the incubators were equipped with a shaker Certomat®R type 886302/4 (B. Braun Biotech International GmbH, Melsungen, Germany)
Clean bench: Scanlaf Clean Bench Fortuna 1500 (Labogene APS, Lynge, Denmark) Clean Air CA/RS4 (Clean Air Supplies Deutschland GmbH, Haan, Germany)
Gel documentation system: UV desk 312 nm (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) Camera system of Doc Print II (PEQLAB Biotechnologie GmbH, Erlangen, Germany)
Microscope setup: Leica DMRB microscope with attached Leica DFC425C camera (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and external light source LEj LQ-HXP 120 (Leistungselektronik JENA GmbH, Jena, Germany).
Lyophilisation installation: Vacuum pump RZ5 with control unit Vacuu-Bas DCP3000 (both manufactured by Vacuubrand GmbH + Co., Wertheim, Germany) and attached refrigerated condensation trap KF-2-60 (Bachofer GmbH, Reutlingen, Germany)
Rotary stirrer: RW20 (IKA®-Werke GmbH & CO. KG, Staufen, Germany)

13.2 Kits

Tab. 15. Kits to perform different isolations or assays

Name	Function	Manufacturer
NucleoSpin® Plasmid	Isolation of plasmid DNA	MACHEREY-NAGEL GmbH & Co. KG , Düren, Germany
NucleoSpin® Gel and PCR Clean-up	Purification of PCR products and gel-electrophoretically separated DNA fragments	
NucleoSpin® RNA Plant	Isolation of total RNA	
ABsolute™ QPCR SYBR® Green reaction mixture	Quantitative real-time PCR	Fermentas GmbH, St.Leon-Rot, Germany
CloneJET™ PCR Cloning Kit	Cloning of blunt-end DNA fragments	
ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit	Sequencing of DNA	Invitrogen, Groningen, The Netherlands

13.3 Enzymes

Tab. 16. Applied enzymes for different assays

Enzyme	Function	Manufacturer
RNase A (ribonuclease A)	Degradation of RNA	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
rDNase (recombinant deoxyribonuclease)	Degradation of genomic DNA	MACHEREY-NAGEL GmbH & Co. KG , Düren, Germany
FastAP™ (thermosensitive alkaline phosphatase)	Dephosphorylation of cloning vector DNA to prevent re-circularisation	Fermentas GmbH, St.Leon-Rot, Germany
Revert Aid™ Premium RT (reverse transcriptase)	cDNA synthesis	
RiboLock™ RNase Inhibitor	Inhibition of RNase A,B, and C	
Phusion® DNA polymerase (DNA depending DNA polymerase)	Polymerisation of DNA during PCR	
Diverse restriction enzyme	Directed hydrolysis of DNA templates	
Diverse restriction enzyme	Directed hydrolysis of DNA templates	New England Biolabs (NEB) GmbH, Frankfurt, Germany

13.4 Chemicals

Tab. 17. Applied chemicals for different assays

Chemical (p. A. quality if not stated)	Manufacturer
Acetic acid	VWR International GmbH, Darmstadt, Germany
2-mercaptoethanol	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany

Acetosyringone (3, 5-dimethoxy-4-hydroxy-acetophenone)	Sigma-Aldrich Corporation, St. Louis (MO), United States of America
Agar agar Kobe 1	Serva Electrophoresis GmbH, Heidelberg, Germany
Agar agar No. 1614	Merck KGaA, Darmstadt, Germany
Agarose SeaKem®	Biozym Scientific GmbH, Hessisch Oldendorf, Germany)
Agarose TopVision™	Fermentas GmbH, St-Leon-Rot, Germany
Ammonium molybdate ($(NH_4)_6Mo_7O_{24}$ x 4 H_2O)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Ampicillin sodium salt	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
BASTA (glyphosinate ammonium)	Bayer CropScience Deutschland GmbH, Langenfeld, Germany
Betaine	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
Boric acid	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Bromophenol blue	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
$CaCl_2$	Merck KGaA , Darmstadt, Germany
Calcofluor White M2R	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
Carboxin	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
Cefatoxim	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
Chloroform	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
$CoSO_4$ x 5 H_2O	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
CTAB (Cetyltrimethylammonium bromide)	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
DEPC (Diethyl pyrocarbonate)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
D-fructose	Merck KGaA , Darmstadt, Germany
D-glucose monohydrate	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
Diammonium hydrogenphosphate	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Disodium dihydrate	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
DMF (N,N-Dimethylformamide)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
DMSO (dimethyl sulfoxide)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
dNTPS (dATP, dCTP, dGTP, dTTP)	Fermentas GmbH, St-Leon-Rot, Germany
EDTA (Ethylenediaminetetraacetic acid)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Ethanol	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
Ethidium bromide	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
$FeCl_3$ x 6 H_2O	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
Formamide	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany

Gelrite	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
Glycerol	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Glycogen	Fermentas GmbH, St-Leon-Rot, Germany
HCl	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
HygroGold™ (Hygromycin B)	InvivoGen, Toulouse, France
Isopropanol	VWR International GmbH, Darmstadt, Germany
Kanamycin sulphate	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
K_2HPO_4	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Kanamycin sulphate	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
KCl	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
KH_2PO_4	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
KOH	Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany
MES (2-(N-morpholino)ethanesulfonic acid)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
$MgCl_2$	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
$MgSO_4$ x 7 H_2O	Merck KGaA, Darmstadt, Germany
$MnSO_4$ x H_2O	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
MOPS (3-(N-morpholino)propanesulfonic acid)	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
Myo-inositol	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
NaCl	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
NaOH	Merck KGaA, Darmstadt, Germany
Nicotinic acid	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
PEG (Polyethylenglycol) 3350/4000	Serva Electrophoresis GmBH, Heidelberg, Germany
Peptone	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
Phenol (TE-equilibrated, pH 7.8)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Potassium acetate	Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany
Primer	Eurofins MWG GmbH, Ebersberg, Germany
Pyridoxine HCL	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
Rifampicin	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
RNase (Ribonuclease)	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
SDS (Sodium dodecyl sulphate)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Sephadex G-50	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
Silwett L-77	Leu & Gygax AG, Birmenstorf, Switzerland

Sodium acetate	Merck KGaA, Darmstadt, Germany
Spectinomycino HCl x 5 H_2O	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
Streptomycin sulphate	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Sucrose	Sigma-Aldrich Corporation, St.Louis (MO), United States of America
Tetracycline HCL	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
Thiamine HCL	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
Tris (Tris(hydroxymethyl)-aminomethane)	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Triton X 100	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany
Yeast extract	DUCHEFA Biochemie B.V., Haarlem, The Netherlands
$ZnSO_4$ x 7 H_2O	CARL ROTH GmbH & Co. KG, Karlsruhe, Germany

13.5 Supplemental data

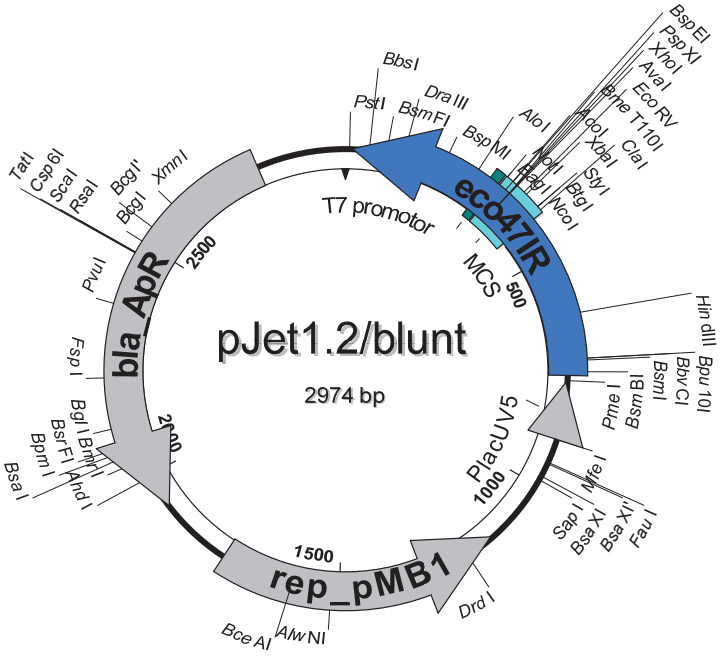

Fig. 65. pJET1.2/blunt vector map
(http://www.fermentas.com/en/-support/technical-reference/phage-plasmid-dna/pbluescriptII) Illustrated are functional elements and recognition sites of selected restriction enzymes; replicon from pMBI plasmid (rep_pMB1), ß-lactamase (bla_ApR); lethality inducing gene (eco47IR), multiple cloning site (MCS), modified P_{lac} promotor for eco47IR expression (PlacUV5), promotor T7 RNA polymerase (T7 promotor).

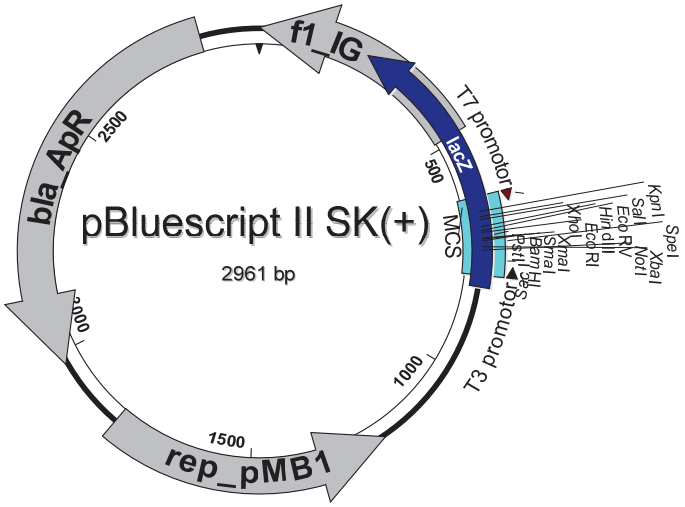

Fig. 66. pBluescript II SK (+) vector map
(http://www.fermentas.com/en/-support/technical-reference/phage-plasmid-dna/pbluescriptII)
Shown are functional elements, selected restriction enzymes; replicon from pmB1 (rep_pMB1), ß-lactamase (bla_ApR), intergenic region of phage f1 (f1_IG), N-terminal fragment of ß-galactosidase (lacZ), multiple cloning site (MCS), promotor T7 RNA polymerase (T7 promotor), promotor of T3 polymerase (T3 promotor).

Tab. 18. Heat (autoclaving) induced pH changes in ammonium phosphate and ammonium chloride supplemented medium
Liquid MMN medium was prepared and different ammonium sources were admixed. Afterwards samples were taken and pH was determined before and after autoclaving to discover putative heat induced pH changes.

$(NH_4)_2HPO_4$ [mM]	pH before autoclaving	pH after autoclaving	NH_4Cl [mM]	pH before autoclaving	pH after autoclaving
MMN	5.44	5.64	MMN	5.44	5.64
0.5	5.94	6.07	0.5	5.33	5.26
1	6.28	6.30	1	5.43	5.32
2.5	6.69	6.66	2.5	5.43	5.32
5	6.96	6.93	5	5.42	5.29
10	7.23	7.17	10	5.39	5.25
25	7.51	7.41	25	5.34	5.17
50	7.65	7.55	50	5.28	5.12
100	7.73	7.63	100	5.19	5.07

Tab. 19. Generated primers for binary vector detection in *Agrobacterium tumefaciens*
Both tabled primers were introduced for detection of transformed agrobacteria through partial amplification (400 bp) of the t-DNA encoded *hph* gene. For annealing of primers, a temperature 53 °C was chosen during standard PCR protocol.

Primer name	Hygro-for	Hygro-rev
Sequence	5'-TCCAGAAGAAGATGTTGGC-3'	5'-CTGCCTGAAACCGAACTG-3'

Tab. 20. Scheme of modifications to induce transformation of *Laccaria* strains till 29.01.2011
Listed are all applied changes to induce biological transformation of fungal strains. Changes in chemicals as well as different incubation durations are given in the row on the left side. Similarly a functional grouping of treatments is visible, for instance to indicate the pre-cultivation or

transformation procedures. The crosses in the rows indicate the applied treatments. The dates of performed transformations are listed in the first line (day of transformation). The grey coloured rows demonstrate the successful transformation attempts. Abbreviations: AS = acetosyringone; OD = optical density, measured at a wavelength of 600 nm. The frequency of transformation indicates the transformed colonies per introduced colonies at this date, while the percentage of overall transformants highlights the percental amount with respect to all 270 generated mutants.

		Day of transformation	28.05.2010	28.05.2010	18.06.2010	30.06.2010	09.07.2010	16.07.2010	20.07.2012	20.07.2010	22.07.2010	22.07.2010	30.07.2010	04.08.2010	24.08.2010	27.08.2010	27.08.2010	04.12.2010	11.12.2010	16.12.2010	11.01.2011	20.01.2011	25.01.2011	29.01.2011
Pre-cultivation	Bacteria	YEB medium	X	X	X	X	X	X	X	X														
		King's B medium									X	X	X	X	X	X	X	X	X	X	X	X	X	X
	Fungi	0.05 % (w/v) glucose																						
		0.5 % (w/v) glucose	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		NH_4Cl (20 mM)			X	X	X	X	X															
		Old fugal start material											X											
		X d prior date at 4 °C																	1.5	2				
		X d prior date on 0.05 % (w/v) glucose	18							3	1	1	3		3									
Transformation	Fungi	Age of fungi [d]	40	18	14	8	10	10	14	14	10	10	10	10	14	14	14	13	10	9	9	12	8	8
		Size of fungal colonies [mm]	30	20	10	< 5	5	5	10	10	5	5	<5	5	10	10	10	<10	5	<5	<5	<10	<5	<6
	Induction	OD (starter-culture)	0.5	0.5	0.5	0.6	0.8	0.6	0.5	0.5	0.8	0.8	0.8	0.8	1.0	0.5	0.5	1.4	1.2	0.8	1.4	0.6	0.95	1.8
		OD-ratio (before and after induction)	0.5/-	0.5/-	0.5/-	0.6/-	0.5/1	0.5/-	0.5/-	0.5/-	0.5/-	0.5/-	0.5/-	0.5/-	0.5/0.88	0.5/0.6	0.5/0.6	0.5/1	0.4/1	0.5/1	0.5/1.8	0.5/1.7	0.45/1.5	0.5/1.2
		Pre-induction in bacterial medium (24 °C; 200 µM AS; slow shaking)	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		Period of induction [h]	4-6	4-6	4-6	4-6	4-6	4-6	4-6	4-6	4-6	4-6	4-6	4-6	6	6	6	4-6	4-6	4-6	9	7.5	6	6
	Dropping solution	Pre-induction culture	X	X	X			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		MMN medium				X	X																	
		5 % (w/v) sucrose				X	X																	
		$MgSO_4$ (10 mM)																						
		Silwett L-77 (0.05 % (v/v))				X	X																	
		Fresh AS (200 µM)				X	X																	
		OD of dropping solution				2.1	1.0								0.88	0.6	0.6	1	1	1	1.8	1.7	1.5	3.4
	Co-cultivation	Agar Kobe 1 = K; Merck 1614 = M	M	M	M	M	M	M	M	M	M	M	M	M	K	K	K	M	M	M	M	M	M	M
		Temperature [°C]	24	24	24	24	24	24	24	24	24	24	24	24	24	24	4+24	24	24	24	24	24	24	24
		$CaCl_2$ (0.1 mM)																						
		0.5 % (w/v) glycerine																						
		Betaine (1 mM)																						

		Period [d]	5	5	5	5	5	6	6	6	5	5	5	5	5	5	1+4	5	5	5	5	5	5	6
		Light exposure (Darkness = D; Light = L)	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D
		Wounding	X	X						X		X				X	X					X		
		MES-KOH 5.2 (10 mM)														X	X							
		0.5 % (w/v) sucrose																						
		0.05 % (w/v) sucrose																						
		0.5 % (w/v) fructose																						
		0.5 % (w/v) glucose	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X							
		0.05 % (w/v) glucose																				X	X	X
		200 µM AS																						
		300 µM AS	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		Bacterial growth during co-culture	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		Fungal growth during co-culture	+	+	+	-	-	+	+	+	+	+	-	+	+	++	++	+	+	+	+	+	+	+
Selection		300 µg/mL hygromycin	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X				
		500 µg/mL hygromycin																			X	X	X	X
Results		Transformed colonies	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	1
		Total colonies	210	210	210	210	70	42	49	49	49	49	56	84	84	84	84	108	108	108	108	135	135	135
		Frequency of transformation [%]	**0.00**	**1.43**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**4.44**	**0.74**
		Percent of overall transformants [%]	**0.00**	**1.11**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**2.22**	**0.37**

Tab. 21. Scheme of modifications to induce transformation of *Laccaria* strains till 03.07.2011

Listed are all chosen changes to trigger the transformation of fungal strains. Changes in media compositions as well as different incubation durations are indicated in the rows on the left side, again besides the grouping of treatments into functional steps. The crosses in the rows indicate the applied treatments. Furthermore, the dates of performed transformations are listed in the first line (day of transformation); the grey highlighted rows demonstrate the successful transformation attempts. Abbreviations: AS = acetosyringone; OD = optical density, determined at a wavelength of 600 nm. The frequency of transformation indicates the transformed colonies per introduced colonies at this date, while the percentage of overall transformants highlights the percental amount with respect to all 270 generated mutants.

		Day of transformation	05.02.2011	12.02.2011	14.02.2011	16.02.2011	17.02.2011	24.02.2011	25.02.2011	28.02.2011	01.03.2011	01.03.2011	09.03.2011	11.03.2011	18.03.2011	21.04.2011	28.04.2011	05.05.2011	13.05.2011	27.05.2011	03.06.2011	11.06.2011	01.07.2011	03.07.2011
Pre-cultivation	Bacteria	YEB medium																						
		King's B medium	X	X	X	X	X	X	X	X	X	X	X	X	X (soya)	X (soya)	X (soya)	X (soya)	X (soya)	X (soya)	X (soya)	X (soya)	X (soya)	X (soya)
	Fungi	0.05 % (w/v) glucose										X	X	X	X									
		0.5 % (w/v)	X	X	X	X	X	X	X	X	X						X							

Selection		Transformation																																			
		Co-cultivation																		Dropping solution							Induction				Fungi						
500 µg/mL hygromycin	300 µg/mL hygromycin	Fungal growth during co-culture	Bacterial growth during co-culture	300 µM AS	200 µM AS	0.05 % (w/v) glucose	0.5 % (w/v) glucose	0.5 % (w/v) fructose	0.05 % (w/v) sucrose	0.5 % (w/v) sucrose	MES-KOH 5.2 (10 mM)	Wounding	Light exposure (Darkness = D; Light = L)	Period [d]	Betaine (1 mM)	0.5 % (w/v) glycerine	$CaCl_2$ (0.1 mM)	Temperature [°C]	Agar Kobe 1 = K; Merck 1614 = M	OD of dropping solution	Fresh AS (200 µM)	Silwett L-77 (0.05 % (w/v))	$MgSO_4$ (10 mM)	5 % (w/v) sucrose	MMN medium	Pre-induction culture	Period of induction [h]	Pre-induction in bacterial medium (24 °C; 200 µM AS; slow shaking)	OD-ratio (before and after induction)	OD (starter-culture)	Size of fungal colonies [mm]	Age of fungi [d]	X d prior date on 0.05 % (w/v) glucose	X d prior date at 4 °C	Old fugal start material	NH_4Cl (20 mM)	glucose
X		+	+	X		X							D	5				24	M	5.5	X	X		X	X		6	X	0.5/1.3	1.0	<3	8			X		
X		++	++		X	X					X		D	5	X			24	M	1.3			X				4	X	0.5/1.5	1.5	5	9					
X		++	++		X	X					X		D	5	X			24	M	1.1			X				8	X	0.4/1.8	1.4	<10	11					
X		++	++		X	X					X		D	5	X			24	M	1.2			X				7.5	X	0.4/1.8	0.9	<3	6					
X		++	++		X	X					X		D	5	X	X		24	K	0.9			X				7	X	0.6/1.65	0.7	<3	7					
X		+	+++	X			X						D	5	X		X	24	M	1.4						X	6.5	X	0.5/1.4	1.0	<5	8					
X		++	++	X		X				X			D	5	X		X	24	K	1.2						X	6.5	X	0.4/1.2	1.0	5	9					
X		++	+++	X				X	X				D	5	X		X	18	K	1.7						X	8	X	0.5/1.70	1.1	5	8					
X		++	++	X			X				X		D	5				18	K	1.4						X	7	X	0.4/1.4	1.2	5	9					
X		++	++	X			X						D	5				24	M	1.4						X	7	X	0.4/1.4	1.2	3	7					
X		+	+	X		X							D	5	X		X	24	K	4.5						X	5	X	0.7/1.2	1.6	3	7					
X		+	+	X		X							D	5	X		X	24	K	5		X			X		7	X	0.5/1.2	1.5	6	9					
X		++	++	X			X						D	5	X		X	24	K	4.5		X			X		7	X	0.4/1.1	0.4	5	8					
X		++	++	X			X						D	5	X		X	24	K	4		X			X		4.5	X	0.5/1.1	1.5	5	8		1			
X		++	++	X			X			X	X		D	5	X	X		24	K	5			X				6.5	X	0.5/1.3	0.9	<4	8					
X		+	+	X		X							D	5				24	K	5.5			X				6	X	0.5/1.8	1.8	4	7					
X		++	+	X						X			D	5	X	X		24	K	5		X	X				6	X	0.3/1.15	1.1	4	7					
X		++	++	X						X	X		D	5	X	X		24	K	5		X	X				6	X	0.45/1.1	1.1	5	8					
X		+	+	X			X			X	X		D	5	X	X		24	K	4.4		X	X				6	X	0.3/0.9	1.3	5	8					
X		++	++	X			X			X	X		D	5	X	X		24	K	4		X	X				6	X	0.3/0.8	1.1	4	8					
X		++	+	X			X			X	X		D	5	X	X		24	K	4.4		X	X				6	X	0.5/1.0	0.9	3	8			X		
X		++	++	X			X			X	X		D	5	X	X		24	K	4.5		X	X				6	X	0.4/1.4	0.9	<10	10			X		

Results																								
	Transformed colonies	17	12	17	5	9	0	0	0	0	0	0	3	1	7	43	0	0	11	0	131	4	0
	Total colonies	180	180	180	135	135	180	180	162	90	270	270	270	270	390	390	336	336	720	720	800	400	400
	Frequency of transformation [%]	**9.44**	**6.67**	**9.44**	**3.70**	**6.67**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**1.11**	**0.37**	**1.79**	**11.03**	**0.00**	**0.00**	**1.53**	**0.00**	**16.38**	**1.00**	**0**
	Percent of overall transformants [%]	**6.30**	**4.44**	**6.30**	**1.85**	**3.33**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**1.11**	**0.37**	**2.59**	**15.93**	**0.00**	**0.00**	**4.07**	**0.00**	**48.52**	**1.48**	**0**

Tab. 22. qPCR template specifications for amplification of the six gene-specific fragments

PCR fragment	TPS1	TP	GS	NT	18S	GP
DNA [ng/mL]	10633.33333	11966.6667	20675	29150	36433.33333	17200
MW [g/mol]	53541.65378	57419.9128	52705.1784	52243.88233	71452.70641	32253.9865
DNA [mol]	1.98599E-13	2.0841E-13	3.9228E-13	5.5796E-13	5.09894E-13	5.3327E-13
DNA [pmol]	0.19859927	0.20840621	0.39227645	0.557960065	0.509894379	0.53326741
Concentration [molecules/µL]	1.19599E+11	1.2551E+11	2.3623E+11	3.36011E+11	3.07065E+11	3.2114E+11

Tab. 23. Dilution series dependent CP values of the six different gene fragments for calibration curves

The six PCR fragments of Tab. 7 were diluted and introduced for qPCR to be able to draw gene-specific calibration curves.

GP fragment			18S fragment			NT fragment		
Dilution	CP	Std. (CP)	Dilution	CP	Std. (CP)	Dilution	CP	Std. (CP)
10^{-3}	10.077	0.112	10^{-3}	9.135	0.318	10^{-3}	8.557	0.098
10^{-4}	13.113	0.186	10^{-4}	12.482	0.543	10^{-4}	11.807	0.095
10^{-5}	16.630	0.148	10^{-5}	16.320	0.507	10^{-5}	15.370	0.130
10^{-6}	19.970	0.095	10^{-6}	19.753	0.629	10^{-6}	18.790	0.095
10^{-7}	23.247	0.067	10^{-7}	21.927	0.643	10^{-7}	22.093	0.081
10^{-8}	25.330	0.325	10^{-8}	25.107	0.741	10^{-8}	25.413	0.263
			10^{-9}	29.283	0.065			
TP fragment			TPS1 fragment			GS fragment		
Dilution	CP	Std. (CP)	Dilution	CP	Std. (CP)	Dilution	CP	Std. (CP)
10^{-3}	12.373	0.133	10^{-3}	10.793	0.151	10^{-3}	9.660	0.098
10^{-4}	16.295	0.021	10^{-4}	14.480	0.121	10^{-4}	13.410	0.061
10^{-5}	20.070	0.313	10^{-5}	18.060	0.139	10^{-5}	17.160	0.070
10^{-6}	23.670	0.478	10^{-6}	21.477	0.248	10^{-6}	20.877	0.133
10^{-7}	26.830	0.035	10^{-7}	24.780	0.193	10^{-7}	24.760	0.398
10^{-8}	28.127	0.127	10^{-8}	27.857	0.178	10^{-8}	27.243	0.208

Tab. 24. Calculated p-values for normalised gene-specific absolute expression results

The numbers of the area-row indicate the outermost (1), middle (2), and central aged hyphal ring (3). The wild type (H70 or respectively H82) expressions of the four genes were used to normalise the mutant-specific expressions revealing expression reductions or inductions in percent. The corresponding p-values are listed, too.

Area	Strain	TPS1	NT	TP	GS	GP
1	S238N-H70	---	---	---	---	---
	TPS1-7003	3.99E-07	5.11E-06	2.03E-06	7.48E-06	1.40E-05
	TPS1-7004	4.63E-04	5.30E-04	3.83E-03	8.00E-04	1.67E-03
	TPS1-7006	2.13E-05	4.63E-06	3.47E-05	4.99E-05	2.52E-04
	TPS1-7007	2.52E-06	2.70E-06	5.28E-06	5.94E-06	3.98E-06
	TPS1-7008	6.84E-07	1.94E-05	4.64E-06	4.20E-06	2.37E-06
	TPS1-7010	3.01E-04	1.25E-04	1.22E-04	3.93E-04	2.12E-04

2	S238N-H70	---	---	---	---	---
	TPS1-7003	4.52E-05	2.78E-04	1.22E-04	1.29E-04	4.60E-04
	TPS1-7004	3.64E-03	1.65E-01	6.06E-03	2.18E-03	1.51E-02
	TPS1-7006	6.34E-04	5.64E-03	4.72E-03	4.89E-03	7.42E-01
	TPS1-7007	5.56E-04	1.11E-02	5.71E-04	2.36E-04	4.53E-04
	TPS1-7008	7.16E-05	9.82E-03	4.21E-04	1.60E-04	3.58E-02
	TPS1-7010	4.74E-04	7.74E-04	6.50E-04	4.03E-04	6.49E-04
3	S238N-H70	---	---	---	---	---
	TPS1-7003	3.47E-03	4.01E-04	1.86E-02	6.93E-07	1.22E-06
	TPS1-7004	9.04E-05	1.19E-04	9.12E-01	9.28E-06	1.33E-05
	TPS1-7006	8.50E-04	7.06E-05	2.04E-03	3.99E-05	4.81E-05
	TPS1-7007	9.67E-04	9.87E-05	4.58E-05	2.19E-04	1.41E-03
	TPS1-7008	2.36E-01	3.77E-04	6.22E-04	1.05E-07	1.20E-07
	TPS1-7010	3.31E-05	1.60E-06	1.30E-06	1.07E-05	1.00E-05
1	S238N-H82	---	---	---	---	---
	TPS1-8204	9.13E-06	1.37E-05	6.13E-06	2.17E-05	3.55E-05
	TPS1-8206	1.24E-04	1.70E-04	4.85E-04	8.47E-05	2.56E-04
	TPS1-8207	2.84E-06	1.09E-04	1.16E-03	1.13E-04	1.96E-04
	TPS1-8208	1.72E-06	1.34E-06	1.60E-06	5.19E-05	9.01E-07
	TPS1-8210	1.52E-05	2.00E-05	4.94E-04	3.95E-04	4.43E-05
2	S238N-H82	---	---	---	---	---
	TPS1-8204	5.90E-06	6.79E-05	2.76E-05	4.25E-05	8.84E-06
	TPS1-8206	1.16E-04	1.49E-04	1.72E-04	2.40E-04	3.84E-05
	TPS1-8207	3.41E-06	1.05E-05	1.08E-04	2.18E-05	5.93E-06
	TPS1-8208	2.54E-02	5.05E-05	5.14E-02	1.16E-04	2.42E-05
	TPS1-8210	8.82E-05	7.46E-05	2.90E-02	1.96E-04	2.39E-05
3	S238N-H82	---	---	---	---	---
	TPS1-8204	4.30E-03	4.76E-04	2.84E-01	2.99E-04	4.60E-04
	TPS1-8206	3.89E-02	3.14E-04	8.71E-05	1.46E-04	2.88E-05
	TPS1-8207	2.94E-03	1.49E-04	3.30E-06	5.88E-06	1.04E-03
	TPS1-8208	9.63E-05	2.30E-01	2.94E-06	1.77E-06	9.01E-07
	TPS1-8210	2.47E-05	1.43E-02	2.13E-08	6.37E-07	1.25E-07

Tab. 25. Obtained fungal sectors after the first selection

Tabled are all performed combinations with the generated putative dikaryotic fungi. Above and at the left side, the selected monokaryons are listed. In the coloured boxes the generated and further cultivated putative dikaryons are indicated with letters (the more letters are listed per cross the more pseudo-dikaryons were isolated). *TPS1*-silenced strains in H70 background were combined with *TPS1*-silenced mutants of strain H82 (light grey boxes) and selected *NT*-silenced strains of H82 (medium grey). Furthermore the same combination was performed with H70 *NT*-silenced mutants and the *TPS1*-silenced H82 (medium grey) and *NT*-silenced H82 (dark grey). However, some combinations were not able to grown on selective plates; these are indicated as boxes without letters.

		TPS1-silenced strain					
		8204	8206	8207	8208	8209	8210
TPS1-silenced strain	7003	A, B	A	A	A, B	A	A, B
	7004	A, B	-	A, B	A, B, C	A, B	A
	7006	A, B	A	A, B	A	A	A
	7007	A, B	A	A, B	A	A, B	-
	7008	A, B, C	A, B	A	A, B, C	A, B	A
	7010	A, B	A	A, B, C	A, B	A	A, B
NT-silenced strain	7002	A, B	A	A, B	A	A, B, C	A
	7005	A	A	A, B	A	A, B, C	A, B
	7006	A, B, C	A, B, C	A, B, C, D	A, B	A, B	-
	7007	A, B, C	A, B, C	A, B, C	A, B, C	A, B, C	A
	7008	A, B, C, D, E, F	A, B, C	A, B, C	A, B	A, B	A, B

	7009	A, B, C, D	A	A, B	A	A, B	A, B
		NT-silenced strain					
		8203	8205	8207	8208	8209	8210
TPS1-silenced strain	7003	A, B	A, B	A, B	A	A	A, B
	7004	A	A, B, C	A	-	A	A, B, C, D
	7006	A	A, B	A	A, B	A, B	A, B,
	7007	A	A	A	A, B, C	A	A, B
	7008	A	A, B	A	A, B	A	-
	7010	A	A	A, B	A, B	A	A, B
NT-silenced strain	7002	A, B, C	A, B	A, B, C	A, B	A, B, C	A, B, C
	7005	A, B	A, B, C	A, B, C	A, B, C	A, B, C	A, B, C
	7006	A	A, B, C	A, B, C	A	A, B	A
	7007	A, B, C	A, B, C	A, B	A, B	A, B, C	A, B, C
	7008	A, B, C	A	A, B, C	A	A	A, A
	7009	A	A	A	-	A, B	A

Tab. 26. Selected fungal mutants after breeding for dikaryon detection through specific staining

Tabled are the randomly selected hybrid strains for microscopy. For three of the analysed isolates, clamp connection formation was not determinable using the applied staining procedure. Regarding to Tab. 25, the light grey boxes indicate the *TPS1-TPS1* double-silenced fungi; the medium grey boxes both mixed *NT-TPS1/TPS1-NT* hybrids and the dark grey boxes the *NT-NT* double-silenced strains. Furthermore, some notes concerning observed phenotypes are listed.

Isolate	Clamp cells	Notes
TPS1-8206 x TPS1-7003A	Yes	Small clamp-connections
TPS1-8206 x TPS1-7004A	Yes	Fine mycelium with small clamp-cells
TPS1-8206 x TPS1-7007B	Yes	Small clamp-cells
TPS1-8206 x TPS1-7008A	Yes	Small clamp-cells
TPS1-8206 x TPS1-7010B	Unsure	Putative heterokaryon, high number of nuclei and both completed and uncompleted clamp-cells
TPS1-8207 x TPS1-7003A	Yes	Big clamp-connections
TPS1-8207 x TPS1-7003B	Yes	Clear clamps
TPS1-8207 x TPS1-7008A	Yes	Very fine hyphae with tiny clamp-cells, but in very high number
TPS1-8207 x TPS1-7008D	Yes	Clear clamp-cells
TPS1-8208 x TPS1-7004A	Yes	Youngest mycelium small in hyphal diameter with finished and unfinished clamps; but older hyphae have definite clamp-cells
TPS1-8208 x TPS1-7004B	Yes	Obvious clamp-cells
TPS1-8208 x TPS1-7008B	Yes	Small clamp-cells
TPS1-8209 x TPS1-7003B	Yes	Clear clamps
TPS1-8209 x TPS1-7007B	Yes	Small clamp-cells
TPS1-8210 x TPS1-7003C	Yes	Very delicate mycelium with finished clamp-cells
TPS1-8204 x NT-7002A	Yes	Clear clamp-cells
TPS1-8204 x NT-7006B	Yes	Definite clamp-cells
TPS1-8204 x NT-7008D	Yes	Obvious clamp-connections
TPS1-8206 x NT-7002A	Yes	Obvious clamp-cells
TPS1-8206 x NT-7005A	Yes	Obvious clamp-connections
TPS1-8207 x NT-7009A	Yes	Clear clamps
TPS1-8208 x NT-7007B	Yes	Explicit mated mycelia
TPS1-8208 x NT-7009B	Yes	Fine and delicate clamp-cells
TPS1-8209 x NT-7002B	Yes	Clear clamps
TPS1-8209 x NT-7005A	Yes	Filigree clamp-cells
TPS1-8210 x NT-7002A	Yes	Small clamp-cells
TPS1-8210 x NT-7006B	Yes	Obvious clamp-cells
NT-8203 x TPS1-7004B	Yes	Small clamp-cells
NT-8203 x TPS1-7010B	Yes	Explicit clamp-connections

NT-8205 x TPS1-7006A	Unsure	Clamp-connections possibly unfinished
NT-8205 x TPS1-7008A	Yes	Obvious clamp-cells
NT-8207 x TPS1-7004A	Yes	Big clamp-connections
NT-8207 x TPS1-7006A	Yes	Very big and explicit clamp-cells
NT-8207 x TPS1-7006B	Yes	Delicate mated mycelium with clamp-cells
NT-8207 x TPS1-7010B	Yes	Obvious clamp-connections
NT-8208 x TPS1-7004B	Yes	Big clamp-cells, but hyphae are somehow disordered
NT-8209 x TPS1-7004C	Yes	Small clamps
NT-8209 x TPS1-7006A	Yes	Very big, much, and obvious clamp-cells
NT-8209 x TPS1-7010C	Yes	Clear clamps
NT-8210 x TPS1-7004A	Unsure	Unsure if clamp-connections are fused
NT-8203 x NT-7005B	Yes	Explicit mated mycelia
NT-8203 x NT-7009A	Yes	Obvious clamp-cells
NT-8205 x NT-7005A	Yes	Clear clamps
NT-8205 x NT-7005A	Yes	Small clamp-cells
NT-8205 x NT-7008A	Yes	Obvious clamp-cells
NT-8205 x NT-7009B	Yes	Explicit and filigree clamp-cells
NT-8206 x NT-7007A	Yes	Obvious clamp-connections
NT-8207 x NT-7009A	Yes	Clear clamps
NT-8208 x NT-7002A	Yes	Small clamp-cells
NT-8208 x NT-7006A	Yes	Clear clamps
NT-8209 x NT-7006B	Yes	Delicate and small clamp-cells
NT-8210 x NT-7002B	Yes	Clear clamps
NT-8210 x NT-7009A	Yes	Big clamp-connections