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Transformation of the mycorrhizal fungus *Laccaria bicolor* by using *Agrobacterium tumefaciens*

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Most boreal and temperate forest trees form a mutualistic symbiosis with soil borne fungi called ectomycorrhiza (ECM). In this association both partners benefit due to nutrient exchange at the symbiotic interface. *Laccaria bicolor* is the first mycorrhizal fungus with its genome sequenced thus making possible for the first time to analyze genome scale gene expression profiles of a mutualistic fungus. However, in order to be able to take full advantage of the genome sequence, reverse genetic tools are needed. Among them a high throughput transformation system is crucial. Herein we present a detailed protocol for genetic transformation of *Laccaria bicolor* by means of *Agrobacterium tumefaciens* with emphasis on critical steps affecting the success and efficiency of the approach.

Ectomycorrhiza (ECM) is a mutualistic symbiosis formed between the fine roots of around 10% of plant families and fungi which predominantly belong to basidiomycetes. This mycorrhizal type dominates in forest ecosystems of boreal and temperate regions as well as in some tropical ecosystems. It is known that in ECM a mutual benefit exists for both partners due to nutrient exchange in symbiotic organs. The fungus receives carbon as photosynthates and the plant receives mainly nitrogen and phosphorus. ECM also improves plant access to soil water resources and increases uptake of other marco- and micronutrients.

Sequencing of the first tree, *Populus trichocarpa*, genome gave access to a full gene data set of a mycorrhizal host.[1] Furthermore, the decision of the Department of Energy Joint Genome Institute (JGI) to sequence the genomes of poplar mycobionts, among them the ECM fungus *Laccaria bicolor*, brought mycorrhizal research into the genomic era.[2] The whole genome sequence of Laccaria has made it possible for the first time to analyze the metabolic traits of a mutualistic fungus specialized to exploit both soil nutrient resources and the symbiotic niche in host plant roots. However, the capacity of ECM research to take full advantage of the genome sequence and genome scale gene expression profiles and turn Laccaria into a model organism faces a serious problem. Detailed gene-to-function studies in ECM depend on the access to reverse genetic tools and these include efficient genetic transformation protocols.

Agrobacterium-mediated transformation (AMT) is a well established technique in plant science which among other benefits avoids the requirement of protoplast production and regeneration. The discovery of the usefulness of AMT in fungi[3] similarly opened a new era for fungal genetics. DNA transfer to fungi apparently works via the same mechanism as in plants, where acetosyringone (AS) induces virulence (*vir*) gene expression, resulting in the excision and liberation of a single-stranded DNA (T-strand) flanked by the direct repeats of the left and right border (LB and RB respectively) of the T-region of Agobacterium Ti-plasmid. This DNA is protected by a protein coat and is transferred via a transmembrane type IV secretion system (T4SS) to the host. Upon import of this deoxyribonucleoprotein structure into the nucleus (T-complex), the T-strand becomes double-stranded (T-DNA) and integrates into the host genome. Whereas the whole machinery for transfer of the T-strand from Agrobacterium to the plant or fungus seems to be provided by the bacterium, integration of the foreign DNA into the genome depends on host factors.[4]

When this project was initiated ECM basidiomycete species had been transformed via AMT with the selection makers *Sh ble* and *hph* conferring resistance to the antibiotics phleomycin and hygromycin B, respectively.[5-7] Unlike filamentous ascomycetes for which several antibiotic and also auxotrophic nutritional selection makers are available, these were the only two selection antibiotics shown to be functional in filamentous basidiomycetes. Auxotrophic nutritional markers are not available for *Laccaria bicolor* and the dikaryotic strain S238N was shown to be naturally resistant to phleomycin. The fungus was however susceptible to hygromycin B which completely inhibited its growth at 100 μg/ml in the culture medium. Hygromycin B is an antibiotic obtained from the genus Streptomyces. It inhibits protein synthesis both in prokaryotes and eukaryotes by interfering with

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Table 1. Modified Pachlewski P5 agar medium

Compound	Per litre
di-NH_4-tartrate	0.5 g
KH_2PO_4	1 g
$MgSO_4$ x $7H_2O$	0.5 g
maltose	5 g
glucose	20 g
thiamine-HCl (added as 1 ml of filter sterilized 1,000x stock solution, stored at 4°C)	0.1 mg
Micronutrients: (added as 1 ml of filter sterilized 1,000x stock solution called kanieltra, stored at 4°C)	
$MnSO_4$ x $4H_2O$	5 mg
H_3BO_3	8.5 mg
$(NH_4)_6Mo_7O_{24}$ x $4H_2O$	0.3 mg
$FeCl_3$	6 mg
$CuSO_4$ x $5H_2O$	0.6 mg
$ZnSO_4$ x $7H_2O$	2.7 mg
agar-agar	20 g
The pH of the medium is adjusted to 5.5 with 1 M KOH and the medium is sterilized by autoclaving for 15 min at 121°C.	

translocation of the ribosome and thus inducing misreading of the mRNA template. The resistance to hygromycin B can be obtained by expressing the hygromycin phosphotransferase gene (*hph*) which enzymatically inactivates the antibiotic. The most common *hph* gene used in transformation of eukaryotes today was originally isolated from *Escherichia coli*.[8]

The susceptibility of *Laccaria bicolor* to hygromycin B allowed us to establish an AMT protocol for this fungus. Several factors, such as fungal starting material, Agrobacterium strain, binary vector, fungal cells/bacteria-ratio, AS induction, co-cultivation time, as well as transformant selection can dramatically influence the success of AMT.[9,10] The AMT of each fungal species is known to require protocol optimization. Nevertheless, AMT of some fungi such as *Aspergillus niger*, *Mucor miehei* and *Tuber borchii* is not reproducible or no stable T-DNA integration has been achieved.[9,11,12]

Agrobacterium-mediated transformation has been shown to function on different intact fungal cell types (mycelium, spores, yeast cells, fruiting body tissue) but their susceptibility can vary depending on the species.[13,14] Also the age of the fungal cells is reported to affect AMT efficiency indicating that active metabolism and high viability of the recipient cells are fundamental for successful gene transfer.[9] The most common starting material for AMT of filamentous ascomycetes is germinated asexual spores due to their easy handling in high quantities. Spores also allow standardization of transformation protocols both for the quantity and the physiological state of the recipient cells. Similar benefits of optimization can be achieved when using yeast cells as well. However, the only possible fungal starting material for *Laccaria bicolor* AMT is vegetative mycelium. Laccaria strains do not produce asexual spores and production of fruiting bodies and thus sexual spores is not feasible under laboratory conditions.

Transformation of *Laccaria bicolor* intact mycelium via Agrobacterium developed in our group[15] is presented here in detail and with special emphasis on critical steps that affect its success. The fungal and bacterial strains used in these studies and their growth conditions and long term storage are also specified.

Fungal and Bacterial Strains and Growth Conditions

This protocol has been optimized for *Laccaria bicolor* (Maire) Orton dikaryotic strain S238N[16] and the sexually compatible monokaryotic strains S238N-H82 and S238N-H107 isolated by in vitro spore germination from one basidiocarp of S238N.[17] The fungal pure cultures were obtained from the Tree-Microbe Interactions Unit, INRA Center of Nancy, France. Fungal strains are cultivated on modified Pachlewski P5 agar medium (**Table 1**) at 22–24°C and covered from light (**Fig. 1A–C**). Both monokaryotic and dikaryotic wild type strains and their derivative transformed strains are stored on modified P5 agar medium in 1.5 ml Eppendorf tubes or 96-well microtiter plates at 4°C and routinely replicated.

Escherichia coli strain TOP10 (Invitrogen) is used for plasmid replication and standard molecular cloning steps. Agrobacterium strains AGL1,[18] and LBA1100,[19] are used for transformation of *Laccaria bicolor* dikaryotic and monokaryotic strains. Transformation and cloning vectors are introduced into electrocompetent bacteria by standard electroporation procedures and the strains are stored as overnight LB cultures in 20% (v/v) glycerol at -80°C (**Fig. 1D**).

Agrobacterium-mediated Transformation (AMT) of *Laccaria bicolor*

Preparation of fungal material for transformation. Fungal colonies for Agrobacterium transformation are produced by inoculating autoclaved 1.5 cm x 1.5 cm cellophane membranes with small fragments of young pre-grown fungal colonies on modified P5 agar medium. The cellophane membranes are boiled before use in a 0.350 g/l EDTA solution for 10 min for eliminating surface impurities and cations and rinsed several times with distilled water before autoclaving in a small volume of ddH_2O. Sterile membranes are placed on fresh agar medium in 9 cm diameter Petri dishes and left open in a laminar hood until the excess water is absorbed. Fungal inocula are let to grow on membranes at 22°C for 5 days in the case of the dikaryotic and the monokaryotic strain H107 and for 7 days in the case of the monokaryotic strain H82. These growth periods produce individual Laccaria colonies of app. 1 cm in diameter.

Preparation of Agrobacterium for Laccaria transformation. Agrobacterium strains (AGL1 and LBA1100) carrying pCAMBIA-based binary vectors (www.cambia.org/daisy/cambia/585.html) are pre-cultivated in 1 ml of LB with 100 μg/ml kanamycin in 1.5 ml Eppendorf tubes at 28°C with 200 rpm agitation for 24 h. Inoculation of these pre-cultures is achieved directly with 5–10 μl of bacteria from the glycerol stocks. This seed-culture is used for inoculating minimum (MIN)-medium (**Table 2**).

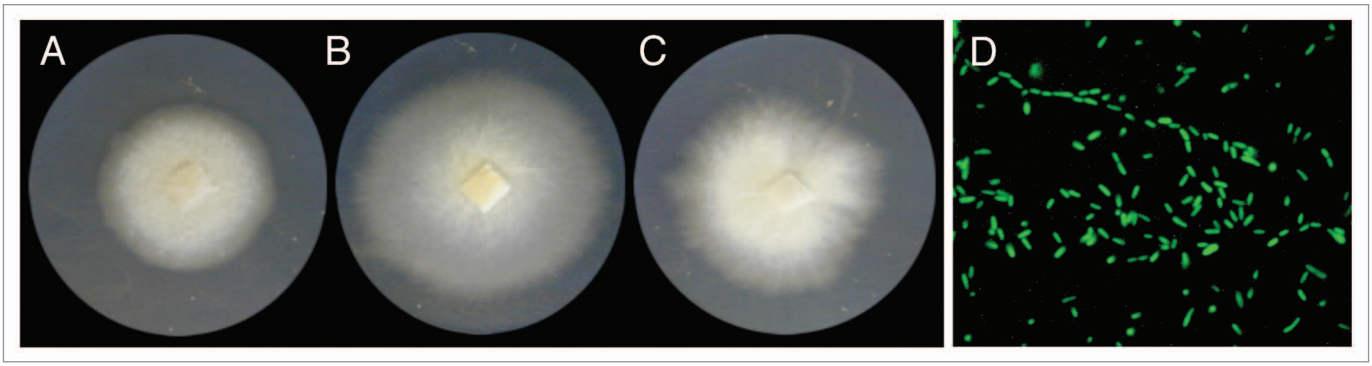

Figure 1. *Laccaria bicolor* (Maire) Orton compatible monokaryotic strains S238N-H82 (A), S238N-H107 (B) and dikaryotic strain S238N (C) grown on modified P5 agar medium at 22°C for 20 days. The diameters of the fungal colonies are (A) 2.4 cm, (B) 3.4 cm and (C) 3.0 cm. (D) Green fluorescent protein-expressing cells of Agrobacterium strain AGL1 growing in IND-medium. Magnification 1,000x.

Fifty milliliter Falcon tubes with 15 ml of MIN-medium supplemented with 100 μg/ml kanamycin are inoculated with 150 μl of Agrobacterium LB seed-culture and cultivated overnight in vertical position at 28°C and 200 rpm. The next day the optical density of MIN-cultures is measured from 1 ml of medium at a wavelength of 600 nm. Absorbance values of 0.2–0.3 are expected after this growth period in MIN-medium and microscopic observation should show actively moving slightly banana-shaped bacteria.

Minimum-Agrobacterium cultures are pelleted for changing the growth medium and initiating *vir*-induction. Cultures are centrifuged at 5,000 rpm for 10 min at 4°C, the supernatant removed and bacteria are re-suspended in the same 50 ml Falcon tube in 15 ml of induction (IND)-medium (**Table 3**) supplemented with 100 μg/ml kanamycin. Bacteria are pre-induced before co-cultivation with the fungus for 6 h at 28°C and 200 rpm. IND-medium initiates *vir*-gene activation by its low pH and the presence of the phenolic inducing compound AS. A concentration of 200 μM of AS is used and the IND-medium is buffered at pH 5.3 with 40 mM MES.

If the OD_{600} of MIN-culture is higher or lower than 0.2–0.3 the initial OD_{600} of IND-culture is adjusted to this range either by removing part of the MIN-culture before centrifugation or by reducing the IND-culture volume.

After 6 h of IND-cultivation the OD_{600} of bacteria is measured again. Induction-growth generally results in OD values of 0.4–0.5 and presents a slightly flocculent appearance. Microscopically, the induced Agrobacterium-culture presents to some extent aggregated, and most of all, less motile bacteria indicating that *vir*-genes have been successfully induced. Final OD_{600} values higher than 0.5 in IND-medium are not recommended. This results in extensive bacterial growth during co-cultivation which can lead, firstly, to too high transformation efficiency, thus compromising isolation of independent transformants and, secondly, to serious problems in elimination of bacteria during the selection steps. Transformation efficiency of Laccaria AMT, when small fungal colonies of vegetative mycelium are used as starting material, is expressed as the number of hygromycin resistant points of growth/number of fungal colonies on 1st selection plates after two weeks of selection.

Table 2. MIN-medium

Compound	Per litre
K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH4)_2SO_4$	1 g
Na-citrate x $2H_2O$	0.5 g
pH ~ 7	
The medium is autoclaved (15 min at 121°C) and stored at RT. The following compounds are added to the autoclaved medium before use:	
1 M $MgSO_4$ (autoclaved, stored at RT)	0.8 ml
1% (w/v) thiamine-HCl (filter sterilized, stored at -20°C)	0.1 ml
20% (w/v) glucose (autoclaved, stored at RT)	10 ml

Table 3. IND-medium

Compound	Per litre
K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH4)_2SO_4$	1 g
Na-citrate x $2H_2O$	0.5 g
glycerol	5 g
MES	8.53 g
The pH is adjusted to 5.3 with diluted (1/10) HCl. The medium is sterilized by autoclaving (15 min at 121°C) and stored at RT	
The following compounds are added to the autoclaved medium before use:	
1 M $MgSO_4$ (autoclaved, stored at RT)	0.8 ml
1% (w/v) thiamine-HCl (filter sterilized, stored at -20°C)	0.1 ml
20% (w/v) glucose (autoclaved, stored at RT)	10 ml
Acetosyringone (AS) (from a fresh 100x stock solution)	200 μM

100x stock solution of AS (20 mM): 1 ml. Four mg of AS are dissolved in 0.1 ml of 100% ethanol at RT. The volume is completed to 1 ml with ddH_2O and the solution is filter sterilized. The AS-solution must be prepared fresh immediately before use.

Table 4. P0.2% co-cultivation agar medium

Compound	Per litre
di-NH_4-tartrate	0.5 g
KH_2PO_4	1 g
$MgSO_4$ x $7H_2O$	0.5 g
glucose	2 g
glycerol	5 g
MES	8.53 g
thiamine-HCl (added as 1 ml of filter sterilized 1,000x stock solution, stored at 4°C)	0.1 mg
Micronutrients: (added as 1 ml of filter sterilized 1,000x stock solution called kanieltra, stored at 4°C)	
$MnSO_4$ x $4H_2O$	5 mg
H_3BO_3	8.5 mg
$(NH_4)_6Mo_7O_{24}$ x $4H_2O$	0.3 mg
$FeCl_3$	6 mg
$CuSO_4$ x $5H_2O$	0.6 mg
$ZnSO_4$ x $7H_2O$	2.7 mg
agar-agar	20 g
The pH is adjusted to 5.3 with 1 M KOH and the medium is autoclaved for 15 min at 121°C	
AS (added to the melted and temperated medium from a fresh 100x stock solution)	200 μM

The corresponding number of plates used for co-cultivation is poured. No kanamycin is added to P0.2% co-cultivation medium.

The co-cultivation conditions. Fungal inocula are prepared for co-cultivation during the 6 h of Agrobacterium induction by switching them, with the help of cellophane membranes, from modified P5 agar medium to P0.2% co-cultivation agar medium supplemented with 200 μM AS.

P0.2% is a derivative of modified Pachlewski P5 agar medium with 0.2% (w/v) glucose, 0.5% (w/v) glycerol and pH buffered at 5.3 with 40 mM MES (**Table 4**).

Approximately 20 pre-grown Laccaria colonies on cellophane membranes can be placed per 9 cm diameter co-cultivation Petri dish. Each fungal colony is inoculated with 20–30 μl of pre-induced Agrobacterium-culture by pipeting bacteria directly onto the colonies. An even distribution and complete soaking of fungal mycelia in bacterial culture is assured by gently whirling and tilting the Petri plates. Also non-Agrobacterium co-cultivation control plates must always be included in the transformation experiment for evaluating the correct functioning of selection conditions afterwards.

The co-cultivation is performed at 22°C and covered from light for a minimum of 3 days. Shorter co-cultivation times do not produce Laccaria transformants but longer times, up to 5 days, increase their number. While longer co-cultivation times increase transformation efficiency of several fungal species the length of co-cultivation can also be one of the fundamental parameters which affect the number of single copy T-DNA transformants arising from the transformation experiment. Longer co-cultivation times are shown to increase multi-copy T-DNA integrations both in *Magnaporthe grisea*[20] and Colletotrichum spp.[21] The maximum 5 days of co-cultivation under the conditions described here result in predominantly singe copy T-DNA integrations in *Laccaria bicolor*.

The growth of bacteria increases during co-cultivation and becomes visible on the cellophane disks generally after 3 days. When excessive bacterial growth is present on plates a shorter co-cultivation time (<5 days) is preferred in order to assure efficient elimination of bacteria during the following selection steps. The growth of fungal colonies, when the maximum number (app. 20 colonies/plate) is used, similarly generates a limiting factor for co-cultivation time. The individual nature of each dikaryotic colony is maintained up to 5 days under the culturing conditions used. While slower growing colonies of the monokaryotic strain H82 could tolerate longer co-cultivation times the excessive bacterial growth often generates a problem after 5 days. The effect of longer than 5 days of co-cultivation on the transgene copy number in monokaryotic transformants has not been studied and is therefore not recommended.

The selection of transformants. The selection of hygromycin B resistant fungal transformants and elimination of Agrobacterium is performed on modified P5 agar medium with 300 μg/ml hygromycin B and 400–600 μg/ml cefotaxime. Even though 100 μg/ml hygromycin B is enough to completely inhibit the growth of both dikaryotic and monokaryotic *Laccaria bicolor* strains, higher hygromycin concentrations are used to ensure the recovery of true-transformed strains. Such a strong selection pressure is used also to compensate long selection times, linked to the slow growth rate of *Laccaria bicolor*, which can lead to reduction of the active selection agent in the medium.

Fungal colonies are shifted from co-cultivation plates, with the help of cellophane membranes, to selection plates, flipped over, pressed against the medium and the membranes are carefully removed. A slight pressure should be applied for assuring that the whole colony makes an even contact with the selection medium. A selection control-plate is prepared with non-Agrobacterium treated Laccaria colonies. The 1st selection is performed at 24°C for two weeks. The selection plates are observed every 2 days both with the naked eye and microscopically for evaluating successful elimination of Agrobacterium, mapping the appearance of hygromycin B resistant fungal points of growth and for proper functioning of the selection pressure. When the dikaryotic Laccaria strain and the monokaryotic strain H107 are used as recipients, transformants start to appear after 5 days of selection and are clearly visible to the naked eye after 8 days. Due to the slower growth rate of the monokaryotic strain H82 a delay of 3–4 days in appearance of transformants is observed.

Transformation efficiencies are calculated after two weeks on 1st selection plates. Appearance of new transformants after this time point is still possible but due to the usually high number of fungal points of growth on selection plates these cannot be mapped with certainty.

Microscopic observation of 1st selection plates has also repeatedly demonstrated that a high number of transiently hygromycin B resistant hyphae are present during the first few days of selection in colonies used for transformation. The growth of these hyphae however ceases and they do not give rise to true hygromycin

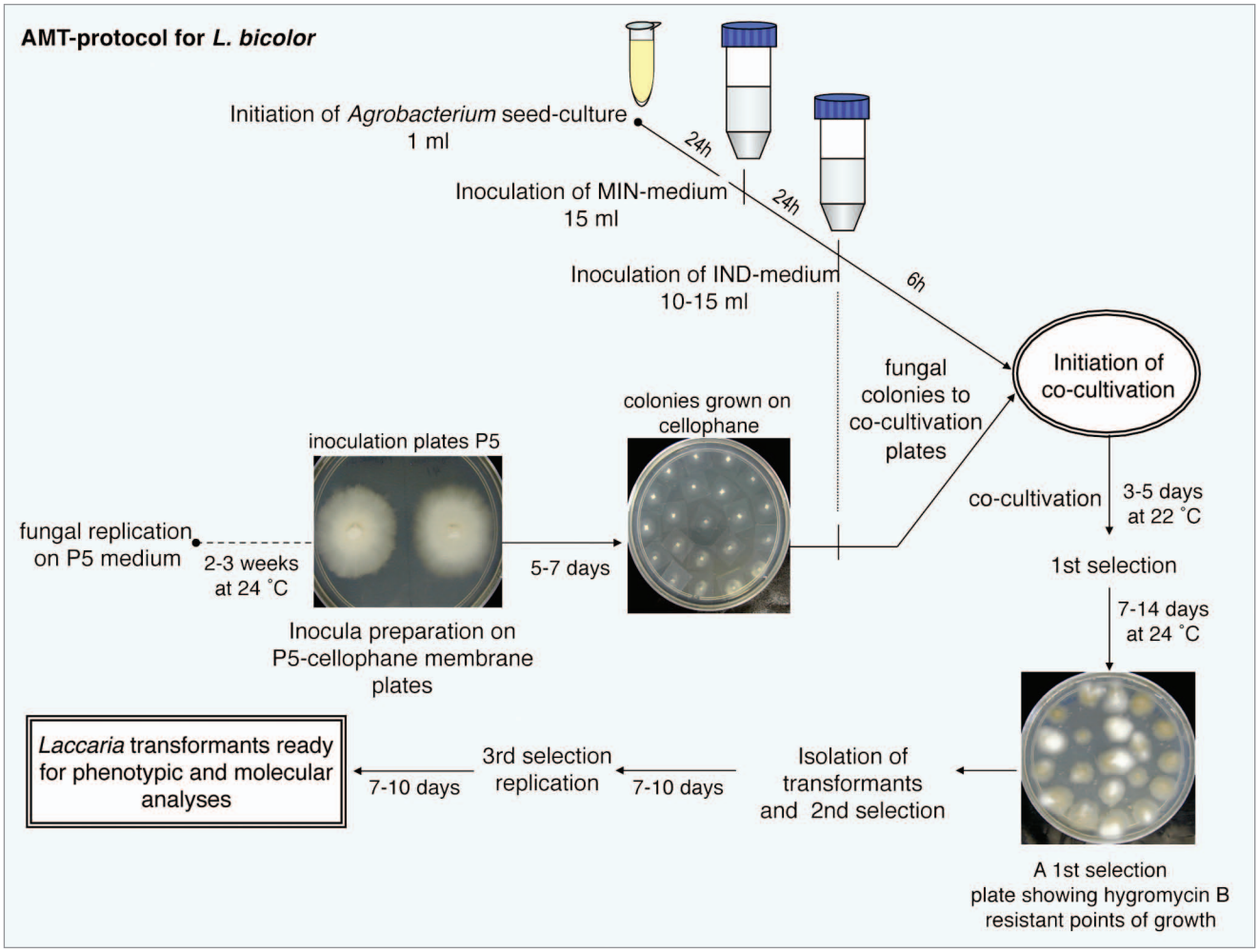

Figure 2. A schematic representation of the *Laccaria bicolor* AMT-protocol.

resistant points of growth. These transiently resistant hyphae most probably represent transient transformation events where no stable genomic T-DNA integration is taking place. Therefore, the entrance of the T-strand into Laccaria cells seems to happen with very high efficiency while the stable T-DNA integration is a more limiting step for AMT of this fungus. A similar type of transient or point-like activation of fungal growth on hygromycin is never observed on Laccaria non-Agrobacterium control selection plates with 300 μg/ml hygromycin B pressure.

If bacterial growth has been excessive during co-cultivation the 1st selection can result insufficient in eliminating Agrobacterium and especially killing bacteria present on top of fungal colonies. Problems in eliminating Agrobacterium may become evident after 3 days of selection when an excessive bacterial growth is observed. These bacteria can be eliminated by performing superficial washes of the 1st selection plates. Washing of the selection plates earlier is however not recommended because colonies under selection can easily detach from the medium. After 3 days of selection hygromycin resistant mycelium generally start to grow attaching the fungal colonies to the agar medium allowing the surface wash. Selection plates are washed in a laminar hood, with liquid modified P5 medium supplemented with 150 μg/ml hygromycin B and 600 μg/ml of cefotaxime. Five milliliters of medium are pipetted on plates covering the whole surface with a thin layer of liquid. The plates are allowed to sit for 5–10 minutes after which bacteria are washed off by carefully pipetting on the problematic zones. The washing-medium is removed and the plates are allowed to dry until no visible humidity is present between colonies (this should take approximately 15 minutes). This treatment normally reduces or completely eliminates the excess bacterial growth during the 1st selection and allows final elimination of Agrobacterium during the 2nd selection.

The second selection is initiated by moving independent hygromycin B resistant points of growth from the 1st selection to fresh 2nd selection plates. These 2nd selection plates have the same medium composition as the 1st selection plates. The independent nature of each hygromycin resistant point of growth is assured by systematic light-microscope observation of their appearance during the 1st selection. Only the points of growth clearly showing a singular origin are used for further selections. Small fragments of mycelia are excised with a sterile needle and placed to 2nd selection for 7–10 days. The replication is repeated for a 3rd selection. If no bacterial growth is observed on the 2nd selection plates the 3rd selection is carried out without cefotaxime pressure.

The hygromycin B concentration is also reduced to 150 μg/ml. The transformants under 3rd selection are allowed to grow for 7–10 days before initiating cultures for cold storage, phenotypic evaluations or mycelia harvest for molecular studies.

A schematic representation of *Laccaria bicolor* AMT protocol is presented in **Figure 2**. Growth and selection times in this figure refer to the dikaryotic S238N or the monokaryotic S238N-H107 strains on modified P5 agar medium. Times are longer when working with the monokaryotic strain S238N-H82 and may vary for other *Laccaria bicolor* strains. The Agrobacterium MIN and IND steps can also be scaled down to 1 ml Eppendorf cultures for easier handling of several transformation trials simultaneously.

Special Notes on Laccaria AMT

The effect of physical harming of fungal colonies on transformation efficiency. Agrobacterium-mediated transformation of Laccaria is successful with the above presented protocol. However, physical harming of the fungal colonies prior to adding the induced bacteria was observed to increase transformation efficiency. When damaging the colonies before co-cultivation transformation efficiencies higher than 130% are generally obtained when working with the dikaryotic fungus and the Agrobacterium strain AGL1 carrying pCAMBIA1300-derived binary vectors.

This physical harming is carried out by repeatedly cutting the colonies with a scalpel, a process which has resulted in doubling the number of hygromycin B resistant transformants detected in the 1st selection. Whether this higher transformation efficiency is due to some direct stimulating effect rising from fungal cell damage and affecting T-strand mobilization, transfer or nuclear integration is not yet known. Plant mutants deficient in the ability to repair single-strand nicks or double-strand breaks have been found to be deficient in T-DNA integration.[22] Cell rupture and activation of nuclear DNA repair mechanisms can thus be involved in the increase of T-DNA integration in the fungal nucleus. The higher transformation efficiency could however also be a result of higher bacterial attraction to the damaged zones or simply due to a better penetration and improved contact of bacteria with somewhat hydrophobic fungal colonies in these wounded sites. An active metabolic state of the target cells is known to be a requirement for successful AMT of fungi. The recovery process of the wounds during Laccaria co-cultivation may also result in higher number of young and metabolically active fungal tips and branches in wounded than in unwounded colonies. This actively growing young mycelium might be more susceptible to AMT via processes related to nuclear replication. Furthermore, single-stranded nicks and double-stranded breaks have been associated with DNA replication.[23]

The effect of selection growth medium on Laccaria AMT. The protocol presented here for Laccaria AMT is optimized for the use of modified P5 agar both as growth and selection medium. Despite the fact that the Laccaria strains used in the current studies prefer the complex modified GPY (glucose-peptone-yeast extract) agar medium. However, the use of GPY agar as a selection medium is incompatible with AMT when working with the dikaryotic strain. The dying process of co-cultivated mycelia leads to liberation of a dark pigment on this medium, a phenomenon not observed when modified P5 agar is used. More importantly, this coloured compound of unknown identity strongly inhibits the growth of transformants. Interestingly, the same compound is not liberated by the monokaryotic strain H82 with which GPY can therefore be used during transformant selection. These observations with Laccaria clearly demonstrate how the general success of fungal AMT may depend on what may otherwise appear as irrelevant factors such as selection medium composition and how a strain-dependent response to these protocol variations can also vary.

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