Agrobacterium-mediated transformation of the filamentous fungus *Aspergillus awamori*

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Many transformation methods have been developed to introduce DNA into filamentous fungi. One of these methods is *Agrobacterium*-mediated transformation (AMT). Here, we describe an efficient protocol for AMT of *Aspergillus awamori*. This protocol has been used to determine the function of *Agrobacterium* virulence genes during AMT, to identify factors influencing transformation frequencies, to generate insertional mutants and to generate *A. awamori* gene knockout transformants. This protocol in not only applicable to *A. awamori*, but can be used as a more general guideline for AMT of other filamentous fungi. Conidiospores are incubated with induced *Agrobacterium*, and, after a cocultivation and selection period, hygromycin-resistant transformants are obtained with a frequency of 200–250 transformants per 1 × 10^6 conidiospores. Using this protocol, transformants can be obtained within 10–12 d.

INTRODUCTION

Filamentous fungi comprise a large group of lower eukaryotes. Most of the filamentous fungi, including many *Aspergillus* species, are saprophytes, which means that these fungi grow on dead organic material. They do so by secreting large amounts of various enzymes that break down these biopolymers. Owing to their high secretion capacity, several filamentous fungal species (e.g., *Aspergillus niger*, *A. awamori* and *Trichoderma reesei*) are widely used for homologous and heterologous protein production[1–3]. In addition, filamentous fungi are important model systems to study fundamental cellular and molecular processes. To develop filamentous fungi as hosts for enzyme and metabolite production, DNA transformation methods had to be developed that allowed molecular engineering approaches to further optimize these production processes. As a result of this need, several different transformation systems for filamentous fungi have been published over the years, such as $CaCl_2$/PEG-mediated transformation, lithium-acetate treatment, particle bombardment and electroporation[4,5]. The advantages and limitations of the different transformation methods for filamentous fungi have recently been reviewed[6].

In 1998, a method for the transformation of filamentous fungi was introduced that was derived from the successful plant transformation system based on the soil bacterium *A. tumefaciens*[7]. *A. tumefaciens* has the ability to transfer DNA (the so-called T-DNA), which is located between two direct repeats (known as the left and right border), to its host. Once inside the host, the T-DNA is targeted to the nucleus where it randomly integrates into the host genome[8–10]. The number of fungi that have been transformed by *A. tumefaciens* has been steadily growing since the initial publication of the method, and *Agrobacterium*-mediated transformation has been shown to be an excellent alternative for conventional fungal transformation methods[11,12].

Overview of *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a plant pathogen capable of causing crown gall tumors on plants by transferring a part of its DNA (T-DNA), located on a tumor-inducing (Ti) plasmid, through a type IV secretion system to the host. Genes that are naturally located on this T-DNA encode enzymes to influence the production of plant growth regulators, and their expression results in uncontrolled growth of the plant cells, giving rise to crown gall tumors[13]. This DNA-transferring capacity of *A. tumefaciens* is used for the transformation of fungi. For this purpose, a so-called binary vector system is used, meaning that *A. tumefaciens* contains, besides its chromosome, two plasmids: the Ti plasmid, devoid of its natural T-DNA but containing a virulence region; and a binary vector, containing the T-DNA (**Fig. 1**). The virulence region on the Ti plasmid is composed of a large number of virulence (*vir*) genes that encode for the T-DNA transfer machinery. Proteins encoded by this virulence region are involved in the formation, transport and possibly also integration of the T-DNA. Phenolic compounds, such as acetosyringone (AS), are used to induce the *vir*

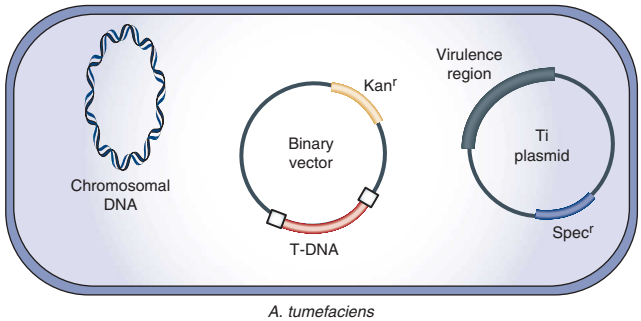

Figure 1 | Schematic overview of the binary vector system for *Agrobacterium*-mediated transformation. The *Agrobacterium* strain used for transformation carries two plasmids. A nononcogenic disarmed tumor-inducing plasmid (Ti plasmid) containing the virulence (*vir*) genes, but lacking the T-DNA region. The T-DNA region is present on a second plasmid, the binary vector. The selection marker in the binary vector used in this study is the kanamycin resistance gene (kan^r) (**Table 2**). DNA sequences to be transformed are cloned between the left and right border sequences of the T-DNA region and transferred to the host. The Ti-plasmid used for *A. awamori* described in this protocol is (pTiB6), which contains the spectinomycin-resistance gene (spc^r)[37].

genes and thus the T-DNA transfer machinery. The binary vector contains the T-region and is surrounded by a 24-bp border repeat, which is the *cis*-acting signal for the DNA delivery system. After induction of the T-DNA transfer machinery, any DNA placed between these border repeats is transferred as a single-stranded DNA molecule to the host where it will randomly integrate into the host genome[8,14–16].

Advantages, limitations and applications of *Agrobacterium*-mediated transformation

The AMT method results in higher transformation frequencies for most fungi when compared with conventional methods. In some cases, fungi recalcitrant to conventional transformation methods can be transformed by AMT[17–21], although it should be noted that not every fungal species can be successfully transformed by *A. tumefaciens*[22,23]. Another key advantage of AMT is that diverse starting materials can be used, such as conidiospores, thereby eliminating the need to generate protoplasts[11]. The efficient generation of protoplasts is often difficult and influenced by several factors, including the growth conditions of the fungal mycelium (medium composition, time of cultivation), and is influenced by strain background or mutants derived from a parental strain. Moreover, the enzymes used to prepare protoplasts can be prone to batch variation, meaning that the protoplasting protocol must be optimized for each enzyme batch. Another major advantage of AMT is that T-DNA is an efficient substrate for homologous recombination, leading to relatively high gene-replacement frequencies[24–28]. Finally, as integration in fungi appears to occur predominately as a single copy, at random and in a nonsequence specific manner when there is no homology between the T-DNA and the host genome, this transformation system is highly suitable for insertional mutagenesis and has been used to generate large insertional mutant collections[29–31]. AMT is less suitable for generating strains for high protein production, due to predominantly single-copy T-DNA integration; multiple gene copies are usually required for higher expression levels. However, multiple gene/T-DNA copies can be forced by introducing a large T-DNA molecule containing multiple gene copies or by changing the cocultivation conditions and/or selection marker[32,33].

Factors influencing transformation efficiency

Optimization of AMT of *A. awamori* and *A. niger*[11,34] revealed several factors influencing transformation efficiency.

AS concentration. The addition of AS during the cocultivation period is essential for the transformation of *A. awamori*, indicating that the induction of the *vir* genes is necessary for T-DNA transfer[34] (**Fig. 2**). In the described protocol, AS is also included during the precultivation of *A. tumefaciens*, but its addition at this step is optional. It has been reported that for some fungi the addition of AS during preculture does not affect the number of transformants obtained, whereas for other fungi the omission resulted in lower transformation frequencies (for review, see ref. 11).

Starting material. Various starting materials, such as conidiospores, protoplasts or germinated conidiospores, can be transformed by *A. tumefaciens*. In the case of AMT of *A. awamori*, a high and reproducible transformation frequency is obtained when conidiospores are used as starting material. To transform other fungi, alternatively, protoplasts or germinated conidiospores

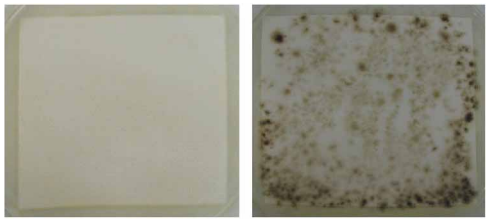

Figure 2 | *Aspergillus awamori* transformation through *Agrobacterium tumefaciens* requires induction of the *vir*-genes by AS. *A. awamori* conidiospores were cocultivated for 3 d at 22.5 °C with *Agrobacterium* LBA1100-pUR5750 without AS (negative control, left panel) or with AS (right panel). AS induces expression of the *vir* genes, which is necessary for DNA transfer. *A. awamori* transformants are selected on MM containing hygromycin (fungal selection marker) and cefotaxim (to prevent *A. tumefaciens* growth on the transformation plate).

can be used; however, the disadvantages of these starting materials for *A. awamori* are that: (i) the protoplasting requires the usage of expensive cell-wall-degrading enzymes and the use of protoplasts does not increase the transformation efficiency compared with conidiospores as starting material[7,35] and (ii) the usage of germinated spores leads to high fungal background growth and to reduced transformation frequencies (C.B.M., unpublished data).

***Agrobacterium tumefaciens* strain.** Various *Agrobacterium* strains have been successfully used to obtain *A. awamori* transformants. In our hands, the use of strain LBA1100 (C58 chromosomal background) and disarmed tumor-inducing plasmid pTiB6 resulted in the highest transformation frequencies for *A. awamori* and *A. niger*, although with different cocultivation conditions. Alternative *Agrobacterium* strains that can be used are, for example, A348, LBA1119 (EHA105) or LBA1126—all strains with a C58 chromosomal background, but a different disarmed Ti plasmid (**Table 1**). For each strain, the optimal cocultivation conditions to obtain a high transformation frequency have to be determined empirically.

Ratio of *Agrobacterium*:spore concentration. Of great influence on the transformation efficiency is the initial amount of fungal material and bacterial cells used in the transformation. An optimal balance between the concentration of fungal and bacterial cells should be experimentally determined for every fungal–*Agrobacterium* combination used to avoid high background growth of the fungus or *Agrobacterium* strain and to obtain optimal transformation efficiency. Initially 1:1, 1:2, 1:3, and 2:1 and 3:1 ratios (fungal spores to bacterial cells) should be tried to determine the effect of different ratios.

Cocultivation condition. A critical step in the transformation procedure is the cocultivation time and temperature. The number of transformants obtained at temperatures between 20 and 28 °C and incubation times between 16 and 96 h has been determined for AMT to *A. awamori*[34]. Lower temperatures are usually beneficial for *A. tumefaciens* to transfer its T-DNA (20–25 °C), but an increased cocultivation incubation time may be required to achieve optimal transformation efficiency. A suboptimal cocultivation period and temperature can lead to reduced transformation efficiency and to *Agrobacterium* or fungal background growth. For *A. awamori* transformations, an optimal cocultivation condition normally lies between an incubation time of 2–3 d and a temperature of 22.5 or 25 °C (ref. 34).

TABLE 1 | *Agrobacterium* strains and Ti plasmids used for *Agrobacterium*-mediated transformation of *A. awamori*.

Agrobacterium strain	Chromosomal background[c]	Ti plasmid	Antibiotic selection marker on Ti plasmid[d]	Reference
LBA1100[a]	C58 (rifampicin resistant)	pTiB6 ΔT-DNA	Spectinomycin	37
LBA1119[a] (EHA105)	C58 (rifampicin resistant)	pTiBo542 ΔT-DNA	None	38
LBA1126[a]	C58 (rifampicin resistant)	pTiB6 ΔT-DNA VirG I77V VirA-TAR	Spectinomycin, carbenicillin	39
LBA4404[a]	Ach5 (rifampicin resistant)	pTiAch5 ΔT-DNA	Streptomycin	40
A348[b]	C58 (rifampicin resistant)	pTiA6NC	None	41

[a]*Agrobacterium* strains containing nononcogenic, disarmed Ti plasmid, lacking the T-DNA region. [b]A348 contains a wild-type Ti plasmid. [c]Rifampicin is included when culturing *Agrobacterium* to prevent contamination. [d]The antibiotic selection for the Ti plasmid (e.g., spectinomycin) is not normally included in the culturing medium as the plasmid is very stable. Glycerol stocks have been made from *A. tumefaciens* strains in the presence of spectinomycin.

MATERIALS

REAGENTS

- *Aspergillus awamori*, e.g., CBS115.52 (CBS)
- *Agrobacterium* strain, e.g., LBA1100, carrying a binary vector harboring the gene of interest and a selectable marker such as for hygromycin resistance. Other *Agrobacterium* strains, such as LBA1119, LBA1126 or LBA4404, can also be used (see **Tables 1** and **2**).
- Acetamide (CH_3CONH_2; Sigma-Aldrich, cat. no. A0500-500g) **! CAUTION** Carcinogenic. Irritant to eyes and skin, harmful if inhaled or ingested. Can form explosive–air mixtures. Wear suitable protective gear.
- AS (3′,5′-dimethoxy-4′-hydroxyacetophenone, $HOC_6H_2(OCH_3)_2COCH_3$; Sigma-Aldrich, cat. no. D134406) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Ammonium nitrate (NH_4NO_3; Merck, cat. no. A4455188) **! CAUTION** Harmful if swallowed. Irritant to eyes, skin and respiratory tract. Wear suitable protective gear. Hazardous oxidizing agent that can react violently with other incompatible materials.
- Bacto agar (Difco, cat. no. 214010)
- Bacto tryptone (Roth, cat. no. 8952.3)
- Boric acid (H_3BO_3; Sigma-Aldrich, cat. no. B9645)
- Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$; Merck, cat. no. 1.02398.1000) **! CAUTION** Do not breathe dust, avoid contact with skin or eyes.
- Casamino acids (Difco, cat. no. 223050)
- Cesium chloride (CsCl; Sigma-Aldrich, cat. no. C4036-250g)
- Cefotaxim (Duchefa, cat. no. C0111.0005)
- Cobalt (II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$; Sigma-Aldrich, cat. no. C8661-100g) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Copper (II) sulfate ($CuSO_4 \cdot 5H_2O$; Sigma-Aldrich, cat. no. C8027-500g) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- DMSO ($(CH_3)_2SO$; Sigma-Aldrich, cat. no. D5879) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- EDTA ($C_{10}H_{14}Na_2O_8 \cdot 2H_2O$; Merck, cat. no. 1.08418.1000)
- Glucose ($C_6H_{12}O_6$; Merck, cat. no. 1.08337.5000)
- Glycerol >99% (Merck, cat. no. 1.04094.1000) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Hybond N+ nylon membrane (Amersham, cat. no. RPN 303B)
- Hydrochloric acid (HCl; Merck, cat. no.100317) **! CAUTION** Causes severe burns. Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective clothing, gloves and eye protection.
- Hygromycin (Calbiochem, cat. no. 400051) **! CAUTION** Toxic, wear suitable protective clothes and gloves, face and eye protection.
- Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$; Sigma-Aldrich, cat. no. F8633-250g) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Kanamycin monosulfate ($C_{18}H_{36}N_4O_{11} \cdot H_2SO_4$; Duchefa, cat. no. K0126) **! CAUTION** Toxic, wear suitable protective clothes and gloves, face and eye protection. Do not breathe dust.
- Magnesium sulfate ($MgSO_4 \cdot 7H_2O$; Sigma-Aldrich, cat. no. M2643-500g)
- Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$; Sigma-Aldrich, cat. no. M5005-100) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Manganese (II) sulfate monohydrate ($MnSO_4 \cdot H_2O$; Sigma-Aldrich, cat. no. 221287-100g)
- (2-*N*-morpholine)-ethane sulfonic acid (MES; Sigma-Aldrich, cat. no. M8250-250g) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Oxoid agar bacteriological (Agar No. 1) (Oxoid, cat. no. LP0011-500g)
- Phleomycin (Invivogen, cat. no. ant-ph-10p) **! CAUTION** Toxic, wear suitable protective clothes and gloves, face and eye protection.
- Potassium chloride (KCl; Merck, cat. no. 1.04936.1000) **! CAUTION** Harmful if swallowed. Irritant to eyes, skin and respiratory tract. Wear suitable protective gear.
- Potassium dihydrogen orthophosphate (KH_2PO_4; Merck, cat. no. 1.04877.1000)
- Dipotassium hydrogenphosphate (K_2HPO_4; Merck, cat. no. 1.05099.1000)
- Potassium hydroxide (KOH; Sigma-Aldrich, cat. no. P5958-1kg) **! CAUTION** Causes severe burns. Avoid contact with skin and eyes. Wear suitable protective clothing, gloves and eye protection.
- Rifampicin (Duchefa, cat. no. R0146) **! CAUTION** Toxic, wear suitable protective clothes and gloves, face and eye protection. Do not breathe dust.
- Sodium chloride (NaCl; Roth, cat. no. 3957.1)
- Sodium hydroxide (NaOH; Merck, cat. no. 1.06498.1000) **! CAUTION** Causes severe burns. Avoid contact with skin and eyes. Wear suitable protective clothing, gloves and eye protection.
- Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$; Sigma-Aldrich, cat. no. S6646)
- Sodium nitrate ($NaNO_3$; Roth, cat. no. A136.2) **! CAUTION** Harmful if swallowed. Irritant to eyes, skin and respiratory tract. Wear suitable protective gear. Hazardous oxidizing agent that can react violently with other incompatible materials.
- Spectinomycin (Duchefa, cat. no. S0188-25) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Tris ($H_2NC(CH_2OH)_3$; MP, cat. no. 819623) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Uridine (Calbiochem, cat. no. 6680) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.
- Yeast extract (Roth, cat. no. 2363.2)
- Zinc sulfate ($ZnSO_4 \cdot 7H_2O$; Sigma-Aldrich, cat. no. Z0251) **! CAUTION** Irritant to eyes and skin, harmful if inhaled or ingested. Wear suitable protective gear.

EQUIPMENT

- Sterile large Petri plates 15 cm (Sarstedt, cat. no. 82.1184)
- Sterile tweezers (VWR, cat. no. 232-2200)
- Glass spreader (VWR, cat. no. 231-2183)
- Centrifuge (Beckman, Allegra 6R)
- Light microscope (Zeiss)
- Eppendorf centrifuge (Eppendorf, cat. no. 5415D)
- Biohazard hood for *Agrobacterium* and fungal handling
- Incubator for *Agrobacterium* and fungal plates (temperature-controlled) (Heraeus, cat. no. B6120)
- Shaker incubator for *Agrobacterium* and fungal culture (temperature-controlled) (New Brunswick Scientific, Innova 4330)
- Spectrophotometer (Hitachi, cat. no. U-2000)
- Hemocytometer (Bürker-Türk)
- Miracloth (Calbiochem)

TABLE 2 | Binary vectors with different fungal selection markers for *Agrobacterium*-mediated transformation of *A. awamori* or *A. niger*.

Binary vector	*Agrobacterium* selection marker	Fungal selection marker[a]	Selection	Fungal selection medium and remarks
pUR5750[7]	Kanamycin	*hph*	Hygromycin resistance	MM containing 100 μg ml^{-1} hygromycin
pTAS5[7]	Kanamycin	*hph*	Hygromycin resistance	MM containing 100 μg ml^{-1} hygromycin
pTAS10[7]	Kanamycin	*hph*	Hygromycin resistance	MM containing 100 μg ml^{-1} hygromycin, T-DNA rescue from transformants possible due to the presence of pUC9 on the T-DNA
pSDMAmdSΔBB[33]	Kanamycin	*amdS*	Acetamide utilization	Acetamide medium
pUR5750AmdS[33]	Kanamycin	*hph* and *amdS*	Hygromycin resistance and acetamide utilization	MM containing 100 μg ml^{-1} hygromycin or acetamide medium, double selection possible
pSDMBLE (unpublished vector)	Kanamycin	*BLE*	Phleomycin resistance	MM containing 100 μg ml^{-1} phleomycin and 0.1 M Tris-HCl, pH 8.0.
pCM1 (unpublished vectors)	Kanamycin	*pyrG*	Uridine prototrophy	Cocultivation on IM containing 1 mM uridine and selection on MM without uridine

[a]The hygromycin and phleomycin fungal resistance cassettes consists of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter and *A. nidulans trpC* terminator. The *A. nidulans amdS* and *A. oryzae pyrG* selection markers contain their endogenous promoters and terminator sequences.

REAGENT SETUP

Hybond N$^+$ filters Filters of the appropriate size are cut from the Hybond N+ nylon membrane roll and sandwiched between two Whatman 3-mm papers. The filters are then stacked and, to keep them dry, wrapped in aluminum foil before autoclaving for 20 min at 121 °C.

Growth media

- **Fungal complete medium (CM).** To 20 g of Bacto agar, add water to make up a total volume of 897 ml; autoclave. Add 20 ml of ASP+N, 20 ml of 50% (wt/vol) glucose, 2 ml of 1 M $MgSO_4$, 1 ml of trace elements, 10 ml of casamino acids and 50 ml of yeast extract to make 1 liter of CM medium. Add uridine (10 mM final concentration) when working with a *pyrG* mutant.
- **Fungal minimal medium (MM).** To 20 g of Bacto agar, add water to make up a total volume of 957 ml; autoclave. Add 20 ml of ASP+N, 20 ml of 50% (wt/vol) glucose, 2 ml of 1 M $MgSO_4$ and 1 ml of trace elements to make 1 liter of MM medium. If required, add antibiotics when the medium has cooled down to 60 °C.
- **Acetamide medium.** To 20 g of Oxoid agar, add water to make up a total volume of 927 ml; autoclave. Add 20 ml of ASP−N, 20 ml of 50% (wt/vol) glucose, 2 ml of 1 M $MgSO_4$, 1 ml of trace elements, 10 ml of 1.5 M CsCl and 10 ml of 1 M acetamide to make 1 liter of acetamide medium.
- **LC medium (liquid).** Dissolve 8 g of NaCl, 10 g of tryptone and 5 g of yeast extract in water to make up a total volume of 1 liter; autoclave.
- **LC medium (liquid) for *Agrobacterium* suspension.** Dissolve 8 g of NaCl, 10 g of tryptone and 5 g of yeast extract in water to make up a total volume of 1 liter; autoclave. Add 200 μl of 50 mg ml^{-1} kanamycin stock (final concentration 100 μg ml^{-1}) and 200 μl of 10 mg ml^{-1} rifampicin stock (final concentration 20 μg ml^{-1}) to 100 ml of LC liquid medium.
- **LC medium (solid) for *Agrobacterium* plates.** Dissolve 8 g of NaCl, 10 g of tryptone, 5 g of yeast extract and 15 g of select agar in water to make up a total volume of 1 liter; autoclave. Add 200 μl of 50 mg ml^{-1} kanamycin stock (final concentration 100 μg ml^{-1}) and 200 μl of 10 mg ml^{-1} rifampicin stock (final concentration 20 μg ml^{-1}) to 100 ml of LC solid medium.
- **Induction medium (IM) (liquid).** Add 0.8 ml of K-buffer, 20 ml of MN buffer, 1 ml of 1% (wt/vol) $CaCl_2 \cdot 2H_2O$, 10 ml of 0.01% (wt/vol) $FeSO_4$, 5 ml of trace elements for IM medium, 2.5 ml of 20% (wt/vol) NH_4NO_3, 10 ml of 50% (vol/vol) glycerol, 40 ml of 1 M MES, pH 5.5, and 10 ml of 20% (wt/vol) glucose to 900.7 ml of sterilized water to make up 1 liter of liquid IM.
- **IM (solid).** Dissolve 15 g of bacto agar in water to make up a total volume of 905.7 ml; autoclave. Add 0.8 ml of K-buffer, 20 ml of MN buffer, 1 ml of 1% (wt/vol) $CaCl_2 \cdot 2H_2O$, 10 ml of 0.01% (wt/vol) $FeSO_4$, 5 ml of trace elements for IM medium, 2.5 ml of 20% (wt/vol) NH_4NO_3, 10 ml of 50% (vol/vol) glycerol, 40 ml of 1 M MES, pH 5.5, and 5 ml of 20% (wt/vol) glucose to make up 1 liter of solid IM.

Chemical solutions (in alphabetical order)

- **1 M acetamide.** Dissolve 5.91 g of acetamide in water to make up a total volume of 100 ml; filter-sterilize.
- **0.2 M AS.** Dissolve 785 mg of AS in DMSO to make up a total volume of 20 ml; aliquot and store in the dark at −20 °C. ▲ CRITICAL Do not thaw and use an aliquot more than twice, as activity of AS decreases during thawing and freezing. ▲ CRITICAL AS is degraded by light.
- **ASP+N.** Dissolve 26.1 g of KCl (350 mM), 74.8 g of KH_2PO_4 (550 mM) and 297.5 g of $NaNO_3$ (3.5 M) in water to make up a total volume of 1 liter; adjust to pH 5.5 by adding 5 M KOH; autoclave.
- **ASP−N.** Dissolve 26.1 g of KCl (350 mM) and 74.8 g of KH_2PO_4 (550 mM) in water to make up a total volume of 1 liter; adjust to pH 5.5 by adding 5 M KOH; autoclave.
- **1% $CaCl_2$ (wt/vol).** Dissolve 10 g of $CaCl_2 \cdot 2H_2O$ in water to make up a total volume of 1 liter; autoclave.
- **10% casamino acids (wt/vol).** Dissolve 100 g of casamino acids in water to make up a total volume of 1 liter; autoclave.
- **1.5 M CsCl.** Dissolve 25.25 g of CsCl in water to make up a total volume of 100 ml; filter-sterilize.
- **0.01% $FeSO_4$ (wt/vol).** Dissolve 0.1 g of $FeSO_4 \cdot 7H_2O$ in water to make up a total volume of 1 liter; filter-sterilize.
- **20% glucose (wt/vol) and 50% glucose (wt/vol).** Dissolve 200 or 500 g of glucose in water to make up a total volume of 1 liter; autoclave.
- **50% glycerol.** Add 50 ml of glycerol to 50 ml of water to make up a total volume of 100 ml; autoclave.
- **K-buffer.** Add solution 1.25 M KH_2PO_4 to 1.25 M K_2HPO_4 until pH 4.8 is reached.
- **1.25 M KH_2PO_4.** Dissolve 170.1 g of KH_2PO_4 in water to make up a total volume of 1 liter; autoclave.
- **1.25 M K_2HPO_4.** Dissolve 217.7 g of K_2HPO_4 in water to make up a total volume of 1 liter; autoclave.
- **1 M MES.** Dissolve 195.24 g of MES in water to make up a total volume of 1 liter; adjust to pH 5.5 by adding NaOH; filter-sterilize. Solution can be stored for a month in the dark or alternatively aliquoted and frozen at −20 °C.
- **1 M $MgSO_4$.** Dissolve 246.48 g of $MgSO_4$ in water to make up a total volume of 1 liter; autoclave.
- **MN buffer.** Dissolve 30 g of $MgSO_4 \cdot 7H_2O$ and 15 g of NaCl in water to make up a total volume of 1 liter; autoclave.
- **20% NH_4NO_3 (wt/vol).** Dissolve 200 g of NH_4NO_3 in water to make up a total volume of 1 liter; autoclave.
- **Physiological salt.** Dissolve 9 g of NaCl in water to make up a total volume of 1 liter; autoclave.
- **Trace elements for CM medium.** Dissolve 2.1 g of $ZnSO_4 \cdot 7H_2O$ (76 mM), 1.1 g of H_3BO_3 (178 mM), 0.5 g of $MnCl_2 \cdot 4H_2O$ (25 mM), 0.5 g of $FeSO_4 \cdot 7H_2O$ (18 mM), 0.17 g of $CoCl_2 \cdot 6H_2O$ (7.1 mM), 0.16 g of $CuSO_4 \cdot 5H_2O$ (6.4 mM), 0.15 g of $Na_2MoO_4 \cdot 2H_2O$ (6.2 mM) and 5.1 g of EDTA (174 mM) to make up a total volume of 100 ml; autoclave.
- **Trace elements for IM medium.** Dissolve 100 mg of $ZnSO_4 \cdot 7H_2O$, 100 mg of $CuSO_4 \cdot 5H_2O$, 100 mg of H_3BO_3, 100 mg of $MnSO_4 \cdot H_2O$ and 100 mg of $Na_2MoO_4 \cdot 2H_2O$ in water to make up a total volume of 1 liter; autoclave.
- **1 M Tris-HCl.** Dissolve 121.14 g of Tris in water to make up a total volume of 1 liter; adjust to pH 8.0 by adding HCl; autoclave.

- **10% yeast extract (wt/vol).** Dissolve 100 g of yeast extract in water to make up a total volume of 1 liter; autoclave.
- **1 M uridine.** Dissolve 244.2 g of uridine in water to make up a total volume of 1 liter; filter-sterilize.

Antibiotic stock solutions

- **0.2 M cefotaxim stock solution.** Dissolve 0.955 g in water to make up a total volume of 10 ml; filter-sterilize, aliquot and store at −20 °C. (1,000× stock solution).
- **100 mg ml^{-1} hygromycin stock solution.** Dilute 2.6 ml (416 mg ml^{-1}) in water to make up a total volume of 9.9 ml; aliquot and store at −20 °C (1,000× stock solution).
- **20 mg ml^{-1} phleomycin stock solution (Cayla).** Ready to use solution. Store at 4 °C (1,000× stock solution).
- **10 mg ml^{-1} rifampicin stock solution.** Dissolve 250 mg in MeOH to make up a total volume of 25 ml; aliquot and store at −20 °C (500× stock solution).
- **10 mg ml^{-1} spectinomycin stock solution.** Dissolve 250 mg in water to make up a total volume of 25 ml; aliquot and store at −20 °C. (40× stock solution).
- **50 mg ml^{-1} kanamycin stock solution.** Dissolve 1 g in water to make a total volume of 20 ml. Aliquot and store at −20 °C. (500× stock solution).

PROCEDURE

Fungal conidia preparation ● TIMING 5 d

1| Spread an aliquot of a 15% (vol/vol) glycerol *Aspergillus awamori* spore solution on a Petri plate containing CM. Glycerol stocks of spores are stored at −80 °C. While taking an aliquot out, keep the spore stock solution in a −20 °C block to prevent complete thawing. Spore stocks can be refrozen and used again.

2| Incubate the plate for 4 d at 30 °C in the dark, to allow *A. awamori* to sporulate.

3| Harvest the spores from the fungal plate by adding 5 ml of sterile physiological salt and gently rubbing the surface of the mycelium with a glass spreader.

4| Filter the spores through Miracloth into a 15-ml Falcon tube and rinse the cloth with 5 ml of physiological salt.

5| Centrifuge the spores for 10 min at 1,000*g* at room temperature, and discard the supernatant and resuspend the spores in 1 ml of physiological salt.

6| Determine the spore concentration using a hemocytometer (Bürker-Türk) and dilute the conidiospores to a final concentration of 1×10^7 conidiospores per ml in IM.

▲ CRITICAL STEP Always use freshly grown and freshly isolated spores. The use of older spore preparations leads to reduced transformation frequency.

Agrobacterium preparation ● TIMING 5 d

7| Start *Agrobacterium* preparation on the same day as fungal spore preparation. Streak *A. tumefaciens* LBA1100 harboring the binary vector of choice (**Table 2**) on an LC plate containing 20 μg ml^{-1} rifampicin (to prevent contamination by other bacterial strains) and 100 μg ml^{-1} kanamycin (to select for the binary vector). Incubate the plate for 3 d at 28 °C. Note that spectinomycin is not included—the Ti plasmid is very stable, so selection is not necessary.

8| Inoculate a single colony of *Agrobacterium* from the LC plate into 10 ml of LC liquid medium containing 20 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin. Culture at 28 °C with shaking at 250 r.p.m. for 24 h. Again, spectinomycin is not included, as selection for the stable Ti plasmid is not necessary.

▲ CRITICAL STEP Shaking, temperature, proper liquid–air balance and duration of incubation are important for good bacterial growth.

9| Spin down 1.5 ml of the *Agrobacterium* culture in a microcentrifuge for 10 min at 2,400*g* at room temperature. Remove the supernatant and wash the cells by gently resuspending the pellet in 250 μl of liquid IM. Centrifuge for 5 min at 2,400*g* at room temperature and remove the supernatant.

10| Resuspend the pellet in 5 ml of liquid IM containing 5 μl of 0.2 M AS. The culturing of *Agrobacterium* cells in the presence of AS before cocultivation is optional. The presence of AS has been reported to be nonessential in some fungi, but its absence resulted in lower transformation frequencies for others (for review, see ref. 11). Furthermore, the addition of this plant phenolic compound to the *Agrobacterium* preculture has been reported to result in either a decrease or an increase in single-copy T-DNA integration[11].

▲ CRITICAL STEP To determine fungal background growth, Steps 10–14 can be performed without addition of AS to the liquid and solid IM (see **Fig. 2**).

11| Incubate the cultures for 4–5 h at 28 °C with gentle shaking at 100 r.p.m in 100-ml Erlenmeyer flasks.

12| Measure the OD at 600 nm using a spectrophotometer and dilute the culture with liquid IM, if necessary, to an $OD_{600\ nm}$ of ~0.8 (4–5 × 10^8 bacterial cells per ml). Note that the length of the incubation period needed to reach an $OD_{600\ nm}$ of 0.8 depends on the *Agrobacterium* strain used in the experiment.

***Agrobacterium*–fungal cocultivation ● TIMING 3 d**

13| Mix 100 µl of the induced *Agrobacterium* cells with an $OD_{600\ nm}$ ~0.8 (from Step 12) and 100 µl of the fungal conidiospores in a concentration of 1×10^7 spores per ml (from Step 6) in a 1:1 ratio.

▲ CRITICAL STEP The number of transformants obtained is dependent on using an optimal ratio of *Agrobacterium* cells to fungal spores: this ratio needs to be determined empirically for each combination of fungal strain and *Agrobacterium* strain (see also TROUBLESHOOTING section). If alternative ratios are tested, adjust the concentration of bacteria or conidiospores so that a total of 200 µl is spread on the Hybond N^+ filter cocultivation plate.

14| With the use of sterile tweezers, place a sterile 0.45 µM Hybond N^+ filter onto Petri plates (15 cm diameter) containing solid IM and 0.2 µM AS.

▲ CRITICAL STEP For better contact between the medium and the *Agrobacterium*–fungal conidiospore mixture, avoid air bubbles.

15| Pipette 200 µl of the *Agrobacterium*–conidiospore mixture onto Hybond N^+ filter on the solid IM and spread the mixture evenly using a glass spreader.

16| Incubate the plates for 3 d at 22.5 °C in a temperature-controlled incubator.

▲ CRITICAL STEP The number of transformants obtained is dependent on an optimal combination of cocultivation time and temperature; these conditions need to be determined empirically for each combination of fungal strain and *Agrobacterium* strain. During the optimization of the reproducibility of AMT to *A. awamori*, we noticed that a temperature difference of 2.5 °C (20, 22.5, 25 and 28 °C) significantly influenced the number of transformants and background growth[35]. It is advisable to start with a systematic approach by testing a combination of different cocultivation temperatures (between 20 and 28 °C) and different ratios of *Agrobacterium* and conidiospore concentrations as a starting point for optimizing transformation frequencies of other fungi.

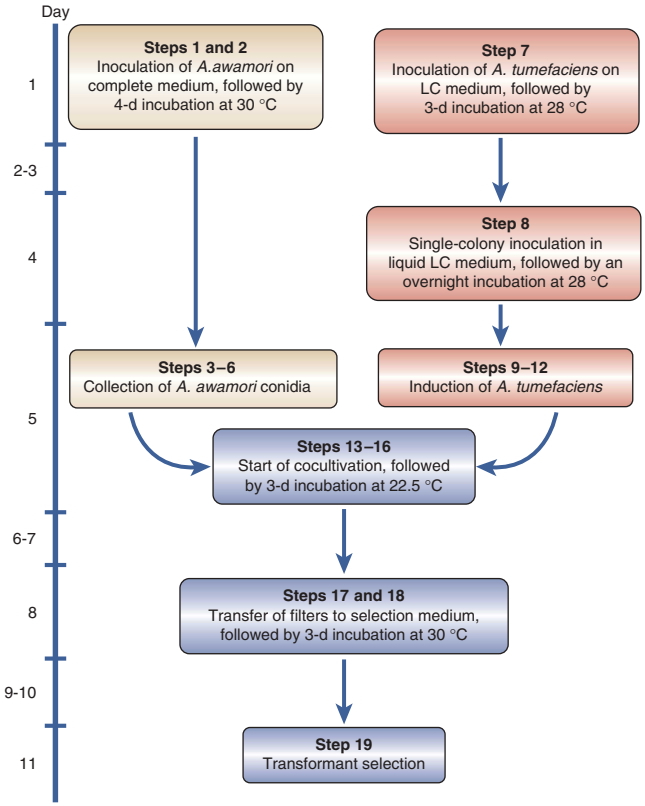

Figure 3 | Flow diagram and time line of the *Agrobacterium*-mediated transformation protocol of *A. awamori*.

Transformant selection ● TIMING 3 d

17| Transfer the Hybond N^+ filters containing the *Agrobacterium*–fungal mixture onto Petri plates containing fungal selection medium (to select for transformants) and 200 µM cefotaxim (to kill the *Agrobacterium*) using sterile tweezers. The type of fungal selection medium depends on the binary vector used (**Table 2**).

18| Incubate the plates for 3 d at 30 °C in the dark until colonies appear.

? TROUBLESHOOTING

19| Transfer the transformants to selection medium for purification and single conidiospore isolation and further characterization.

● TIMING

The entire procedure takes approximately 11 d to complete. A flow diagram outlining the timing of the PROCEDURE can be found in **Figure 3**.

Steps 1–6, fungal conidiospores preparation: 5 d

Steps 7–12, *Agrobacterium* preparation: 5 d

Steps 13–16, *Agrobacterium*–fungal cocultivation: 3 d

Steps 17–19, transformant selection: 3 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
18	High fungal background growth	Conidiospore concentration too high	Reduce the concentration of spores in the cocultivation period
		Cocultivation time too long	Reduce the cocultivation incubation time
		Cocultivation temperature too high	Reduce the cocultivation incubation temperature
		Selection pressure too low	Increase the concentration of the selective agent
			High fungal background growth may also be avoided by pouring the selective medium over the plates as an overlay
	High *Agrobacterium* background growth	*Agrobacterium* concentration too high	Reduce the concentration of *Agrobacterium* in the cocultivation period
		Cocultivation time too long	Reduce the cocultivation incubation time
		Cocultivation temperature too high	Reduce the cocultivation incubation temperature
		Selection pressure too low	In addition to cefotaxim, add carbenicillin (100–500 μg ml^{-1}) or vancomycin (100–800 μg ml^{-1}) to the selection medium
	No transformants or low transformation efficiency	Inappropriate fungal starting material	Try to use germinated conidiospores, protoplasts or mycelium in stead of conidiospores
		Inappropriate *Agrobacterium* strain	Use a different *Agrobacterium* strain
		Inappropriate filters	Change carrier material: alternatives are filter paper, cellophane sheets or PVDF filters
		Suboptimal ratio of fungal conidiospores: *Agrobacterium*	Increase or reduce the *Agrobacterium* or fungal conidiospore concentration
		Suboptimal cocultivation conditions	Increase or reduce the cocultivation time or temperature

ANTICIPATED RESULTS

This protocol has been used to evaluate and identify critical steps in *Agrobacterium*-mediated transformation of filamentous fungi using *A. awamori* as a model organism[11,34,36] and is adapted from de Groot *et al*[7]. Typically, over 200 *A. awamori* transformants per 10^6 conidiospores can be obtained when hygromycin is used as a selection marker. In addition, 60–80% of the transformants carry a single T-DNA insertion. Average transformation frequencies of 0.2, 40 and 80 transformants per 10^6 conidiospores can be obtained when acetamide utilization, uridine prototrophy or phleomycin resistance are used to select for transformants. Transformants with multicopy T-DNA insertions (2–3 copies) are anticipated when the *amdS* gene is used as a selection marker[33]. Omission of AS during the entire procedure can be used as a negative control; no growing colonies should be seen on selection plates when AS is omitted (**Fig. 2**). Suggestions to decrease excessive background fungal growth on the negative control plates (without AS addition during the entire procedure) are given in **Table 3**.

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