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FULL PAPER

Agrobacterium tumefaciens-mediated transformation of the violet root-rot fungus, *Helicobasidium mompa*, and the effect of activated carbon

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Abstract *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been successfully applied to the violet root-rot fungus *Helicobasidium mompa*, which is the causal agent of violet root-rot disease. The *A. tumefaciens* strains carried a binary plasmid vector containing the hygromycin B phosphotransferase gene (*hph*) controlled by the heterologous fungal *Agaricus bisporus* P-*gpd* (glyceraldehyde-3-phosphate dehydrogenase) promoter and the *trpC* terminator. The transformation system was optimized using defined cocultivation conditions. When *H. mompa* strain V17 was cocultivated with *A. tumefaciens* strain AGL-1 using 5% agar, we obtained more hygromycin-resistant colonies than with strains EHA105 or MAFF301222 using 2% agar. In addition, our results suggest that the activated carbon is necessary in ATMT to reduce background growth of *H. mompa*. The presence of the *hph* gene in transformants was detected by polymerase chain reaction (PCR), and single-copy integration of the marker gene was demonstrated by Southern blot analysis. Thus, the ATMT system can be considered a promising tool for insertional mutagenesis studies of *H. mompa*.

Keywords ATMT · Hygromycin B · Insertional mutagenesis

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Introduction

The violet root-rot fungus *Helicobasidium mompa* Tanaka is a commercially important soil-borne root pathogen affecting a wide range of plant species. It is the causal agent of violet root-rot disease, and host plants infected by the fungus quickly wither and die. In Japan, this fungal disease, which spreads rapidly and is very difficult to prevent, has caused great damage to commercially grown grapevines, apple and pear trees, and other crops. Among the few effective control methods for violet root rot are fungicides and biological control. However, long-term prevention of this root disease is difficult. Unfortunately, despite its importance, little is known about its life cycle or control of the disease (Osaki et al. 2002; Aimi et al. 2003a). Valuable knowledge about *H. mompa*'s life cycle and pathogenicity at the molecular level could be gained from genetic studies. However, until recently, no transformation system was available for *H. mompa*. A standard polyethylene glycol-mediated transformation system was used in a gene expression study in white root rot causing *Rosellinia necatrix* (Pliego et al. 2009). However, protoplast-based transformation of *H. mompa* has not been possible because protoplasts have not been successfully generated from *H. mompa*.

Agrobacterium tumefaciens is a pathogenic bacterium that can cause crown gall in plant roots. It is capable of transferring a segment of its Ti plasmid DNA into plant cells, where it is integrated into the host chromosome. By taking advantage of this property of *A. tumefaciens*, *A. tumefaciens*-mediated transformation (ATMT) has been widely used as a transformation technique for plants. ATMT has also been exploited for fungal transformation. *Agrobacterium* is able to deliver its T-DNA into chromosomes of the budding yeast, *Saccharomyces. cerevisiae*

(Bundock et al. 1995; Piers et al. 1996) and diverse filamentous fungi (de Groot et al. 1998; Chen et al. 2000; Malonek and Meinhardt 2001). In addition, ATMT does not require protoplasts and can be used to transform a broad spectrum of starting material. Protoplasts, hyphae, spores, and even blocks of mushroom mycelial tissue (Chen et al. 2000) have been transformed through ATMT. Therefore, ATMT is expected to be an efficient, convenient, and stable transformation technique for fungal species from which it is difficult to make protoplasts. However, it is not clear whether there is an *A. tumefaciens* strain able to infect the particular host fungus because of the strain specificity of *A. tumefaciens*. Therefore, it is necessary to find an *A. tumefaciens* strain that is able to infect the host fungus and examine the optimum conditions for ATMT.

Here we report the successful ATMT of vegetative mycelia of *H. mompa*. Concurrently, we established convenient and efficient ATMT by selecting an *A. tumefaciens* strain and identifying optimum cocultivation conditions for the transformation of *H. mompa*. Moreover, we suggest that activated carbon is necessary for ATMT using *H. mompa*. This is the first report of the genetic transformation of *H. mompa* by applying ATMT, a system that provides a powerful means for genetically manipulating this phytopathogenic fungus.

Materials and methods

Strains and plasmid

The field-isolated strains of *H. mompa* used in this study (Table 1) were originally obtained from Dr. Hitoshi Nakamura (National Institute of Agro-Environmental Sciences, Japan) and were routinely grown on potato dextrose agar (PDA; potato extract, 2% glucose, 2% agar) at 25°C. Mycelia for DNA extraction were grown on a cellophane sheet overlaid on PDA. The *A. tumefaciens* strain EHA105 (Lazo et al. 1991) was provided by Dr. Yoshiyuki Niimi (Prefectural University of Hiroshima, Japan). The *A. tumefaciens* strains AGL-1 (ATCC BAA-101) and MAFF301222 were supplied by the American Type Culture Collection (Krishnamohan et al. 2001) and the National Institute of Agrobiological Sciences (Ohta and Nishiyama 1984), respectively. These *Agrobacterium* strains were routinely grown on Luria–Bertani (LB) agar (1% sodium chloride, 1% tryptone, 0.5% yeast extract, pH 7.0) containing 50 μg/ml kanamycin to maintain the plasmid vector for transformation experiments.

The plasmid vector pBGgHg (Chen et al. 2000) was used for transformation and contains the hygromycin B phosphotransferase gene (*hph*), which is controlled by the glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter from *Agaricus bisporus*. *A. tumefaciens* strains AGL-1, EHA105, and MAFF301222 were transformed by electroporation with constructed binary vectors using a Gene Pulser system (Bio-Rad, Tokyo, Japan) according to the supplier's protocol (Mozo and Hooykaas 1991).

Influence of *H. mompa* on the growth of *A. tumefaciens*

The influence of *H. mompa* on growth of *A. tumefaciens* during cocultivation was examined. *A. tumefaciens* strains AGL-1, EHA105 and MAFF301222 were cocultivated with seven strains of *H. mompa* on malt yeast extract agar

Table 1 *Helicobasidium mompa* and *Agrobacterium tumefaciens* strains used in this study

Strain	Characteristics	Source
H. mompa		
V664	dsRNA free	Dr. Nakamura
V777	dsRNA free	
V17	Totivirus	
V18	Mitovirus	
V70	Partitivirus	
V670	Endomavirus	
V3M	Monokaryon	
A. tumefaciens		
AGL-1 (BAA-101)	AGL0 *recA::bla* pTiBo542ΔT-region Mop^{+} Cb^{R}	American Type Culture Collection (Krishnamohan et al. 2001)
EHA105	A *vir* helper, L,L-succinamopine type, harbors T-DNA deletion derivative of pTiBo542	Dr. Y. Niimi (Lazo et al. 1991)
MAFF301222		National Institute of Agrobiological Sciences (Ohta and Nishiyama 1984)

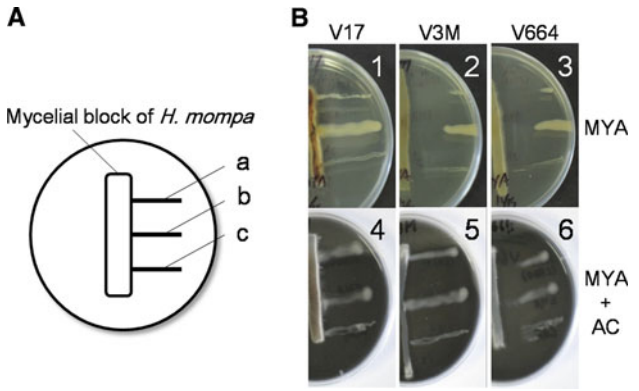

Fig. 1 Influence of *Helicobasidium mompa* on growth of *Agrobacterium tumefaciens*. **a** A rectangular mycelial agar block of *H. mompa* was put in the center of malt yeast extract agar (MYA) medium. A single colony each of *A. tumefaciens* strains AGL-1 (*line a*), EHA105 (*line b*), and MAFF301222 (*line c*) was inoculated onto the block and streaked at a right angle. **b** *A. tumefaciens* strains were cocultivated with *H. mompa* strains V17 (*panels 1*, *4*), V3M (*panels 2*, *5*), and V664 (*panels 3*, *6*) on MYA (*panels 1–3*) or MYA with 0.2% (w/v) activated carbon (AC) (*panels 4–6*). The plates were incubated at 25°C for 3 days

(MYA; 2% malt extract, 0.2% yeast extract, 2% agar, pH 6.5) or induction medium agar [IMA; 10 mM glucose, 2.05 g/l K_2HPO_4, 1.45 g/l KH_2PO_4, 0.15 g/l NaCl, 0.50 g/l $MgSO_4 \cdot 7H_2O$, 0.10 g/l $CaCl_2 \cdot 6H_2O$, 0.0025 g/l $FeSO_4 \cdot 7H_2O$, 0.5 g/l $(NH_4)_2SO_4$, 0.5% glycerol, 40 mM 2-(*N*-morpholino)ethanesulfonic acid, 2% agar, pH 5.3] (Bundock et al. 1995) with or without 0.2% (w/v) activated carbon (AC). A mycelial agar block of *H. mompa* was cut into rectangles and put in the center of the plate. A single colony of three *A. tumefaciens* strains was streaked at a right angle starting from the rectangular block and ending toward the edge of the plate (Fig. 1a). The plates were incubated at 25°C for 3 days.

A. tumefaciens-mediated fungal transformation

The transformation procedure was based on the protocol described by Chen et al. (2000), with some modifications, as follows. Single colonies of *A. tumefaciens* (strains AGL-1, EHA105, and MAFF301222) carrying the plasmid pBGgHg were grown in 10 ml LB broth containing 50 μg/ml kanamycin overnight at 28°C with shaking at 120 rpm. One milliliter of each fresh culture was transferred to 100 ml minimal medium (MM; de Groot et al. 1998) supplemented with kanamycin. The fresh cultures were collected by centrifugation at 3,000*g* for 10 min at 15°C and the pellets were washed with IM. The cells were collected again and suspended in IM to an optical density at 600 nm of 0.5–0.8. The bacterial suspension was incubated for 5–6 h at 28°C under agitation (120 rpm) to preinduce the virulence of *A. tumefaciens*.

The *H. mompa* strains were grown in PD (potato extract and 2% glucose) medium at 25°C for 2 weeks. The culture broth containing the mycelial mat was homogenized with a Nissei Homogenizer (AM-12) at 7,000 rpm for 1 min. The homogenized mycelium and the virulence-preinduced *A. tumefaciens* were mixed and collected by centrifugation. The resultant pellet containing *H. mompa* mycelium and *A. tumefaciens* was transferred onto a cellophane sheet overlaid on IMA medium with different agar concentrations (2% or 5%) containing 200 μM acetosyringone. After 7 days of coculture on IMA medium at 25°C, the cellophane sheets were transferred onto selection medium [SM; PDA containing 60 μg/ml hygromycin B for selection of transformants and 25 μg/ml meropenem to kill *A. tumefaciens* (Cao et al. 2006)].

PCR and Southern blot analysis

Genomic DNA from *H. mompa* strains was isolated using the hexadecyltrimethylammonium bromide (CTAB) procedure as described previously (Aimi et al. 2003b), digested with the appropriate restriction endonucleases, separated by agarose gel electrophoresis, and blotted onto Hybond-N^+ nylon membranes (Amersham Biosciences, Piscataway, NJ, USA). DNA hybridization probes were labeled and detected using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Mannheim, Germany). Procedures for probe labeling, hybridization, and detection were carried out according to the manufacturer's recommendations. To amplify *hph* DNA, which was used both to analyze the transformant strains and as a probe, oligonucleotide primers hph1 (5′-TTC GAT GTA GGA GGG CGT GGA-3′) and hph2 (5′-CGC GTC TGC TGC TCC ATA CAA G-3′) were designed based on the nucleotide sequence of *hph* in pAN26 (Wang and Li 2008). The amplified DNA fragments encoding *hph* were purified with a Wizard SV Gel and Polymerase Chain Reaction (PCR) Clean-Up System (Promega) and used as a DNA template for direct sequencing and as a probe for Southern blot analysis. DNA sequencing was carried out in an ABI 310 Genetic Analyzer (Applied Biosystems) by a chain-termination procedure with a BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) using the hph2 primer.

Effect of AC concentration on transformation efficiency

The effect of AC on transformation efficiency was examined in cocultivation. To determine the efficiency, small mycelial cubes cut with a Pasteur pipette from the margins of young *H. mompa* mycelia were inoculated onto cellophane sheets on PDA (Hanif et al. 2002). After 3 days of incubation at 25°C, the cellophane sheets with the young

mycelial fragments were transferred to IMA medium. These young mycelial fragments were cocultivated by pouring the virulence-preinduced *A. tumefaciens* onto the IMA medium containing the fragments. Three concentrations (0.2%, 0.5%, and 1%) of AC were assessed to determine optimum transformation efficiency. After 7 days of coculture on IMA medium at 25°C, the cellophane sheets were transferred onto SM. Hygromycin-resistant colonies appeared after approximately 4 weeks. These colonies were transferred to and maintained on PDA containing 60 μg/ml hygromycin B.

Results and discussion

Influence of *H. mompa* on growth of *A. tumefaciens*

Assays using a modified agar streak method (Bhaskaran et al. 1974) were carried out on seven strains of *H. mompa* during cocultivation with three strains of *A. tumefaciens* (Table 1). After these plates were coincubated for 3 days, even though all three strains of *A. tumefaciens* appeared on MYA plates without AC, we observed no growth of any *A. tumefaciens* strains around mycelial agar blocks of *H. mompa* except for strain V17 (Table 2, Fig. 1b). This result indicated that all tested strains of *A. tumefaciens* were quite sensitive to *H. mompa* on MYA without AC. However, *A. tumefaciens* colonies were able to grow not only with strain V17 but also with V3M when grown on MYA containing 0.2% (w/v) AC. In cultivation on MYA with the AC, although the mycelium of *H. mompa* became a thick filament, the growth rate was slower than without the AC. The same results were obtained when the assays were performed using IMA with or without AC (Table 2). *A. tumefaciens* made contact with strains V17 and V3M on IMA with AC.

From the result of agar streak assays, we concerned about antibacterial substance of *H. mompa* to *A. tumefaciens* for the ATMT. Some antibiotics, such as helicobasidin, and organic acids as to metabolic products of *H. mompa* had been known (Takai 1966). Helicobasidin is a benzoquinone that inhibits growth of *A. tumefaciens* have also been reported. On the other hand, AC is widely employed in wastewater treatment containing aromatic compounds because of its strong adsorption capacity. We thought that the AC might adsorb those inhibitors, because it has been reported that benzoquinone could be adsorbed on the AC (Minghua et al. 2005). In fact, *A. tumefaciens* strains could grow with *H. mompa* strain V3M on MYA and IMA when AC was added in agar streak assays. This result suggests that those inhibitors were adsorbed onto the surface of the AC.

We believe that it is difficult to transform *H. mompa* using the ATMT system because the influence of *H. mompa* on the growth of *A. tumefaciens* is observed within 2 days. However, our study showed that the effect of AC in cocultivation and ATMT efficiency can potentially be as high in *H. mompa*.

A. tumefaciens-mediated transformation of *H. mompa*

Growth inhibition by hygromycin B of wild-type strains and putative transformants of *H. mompa* was tested using PDA supplemented with various concentrations of hygromycin B. The growth of wild-type strains was extremely inhibited at 50 μg/ml hygromycin B in PDA (data not shown). From this result, selection of transformants was performed at 60 μg/ml hygromycin B in ATMT of *H. mompa.*

To establish whether *A. tumefaciens* transfers T-DNA to *H. mompa*, the binary vector pBGgHg was introduced to three *A. tumefaciens* strains (Table 1). *A. tumefaciens* cells carrying pBGgHg were cocultivated with mycelia of seven *H. mompa* strains. We observed hygromycin-B-resistant colonies of the strain V17 approximately 30 days after transfer to the selection medium. These resistant colonies could grow on PDA containing 80 μg/ml hygromycin B (Fig. 2). On the other hand, no growth of wild-type strain V17 appeared on the same medium. These results suggest that the *gpd* promoter from *A. bisporus* can function in *H. mompa* strain V17. However, we did not obtain resistant colonies from other *H. mompa* strains, suggesting that

Table 2 Influence of *Helicobasidium mompa* on growth of *Agrobacterium tumefaciens*

Medium	*H. mompa* strains						
	V17	V3M	V18	V70	V664	V670	V777
MYA, IMA	+	–	–	–	–	–	–
MYA + 0.2% (w/v) AC, IMA + 0.2% (w/v) AC	+	+	–	–	–	–	–

+ *Agrobacterium* strains could grow around the mycelial agar block of *H. mompa*; – *Agrobacterium* strains could not grow around the mycelial agar block of *H. mompa*

MYA malt yeast extract agar, *IMA* induction medium agar, *AC* activated carbon

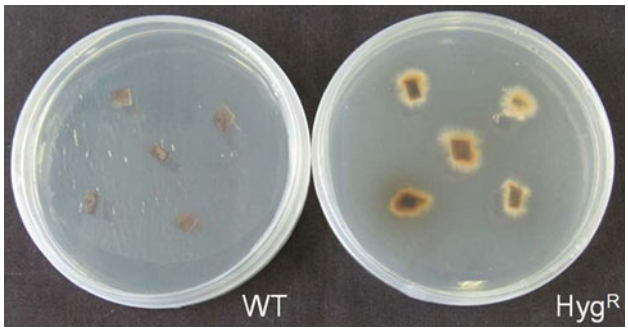

Fig. 2 Example of *Agrobacterium-tumefaciens*-mediated transformation of *Helicobasidium mompa*. *A. tumefaciens* strain AGL-1 containing pBGgHg was cocultivated with mycelia of *H. mompa* strain V17. Shown is the growth of wild-type (*WT*) and putative transformants (*Hyg^R*) of *H. mompa* after 2 weeks on selection medium (SM) containing 80 μg/ml hygromycin B

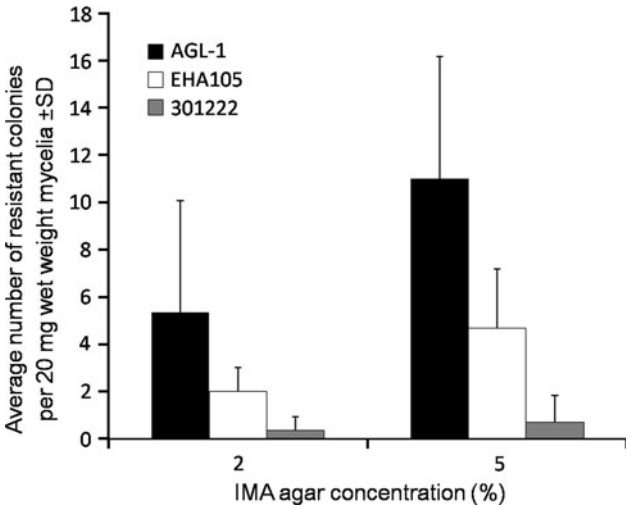

Fig. 3 The effect of various *Agrobacterium tumefaciens* strains and agar concentrations on transformation efficiency of *Helicobasidium mompa* strain V17. Strain V17 was cocultivated with *A. tumefaciens* strain AGL-1 (*black bars*), EHA105 (*white bars*), and MAFF301222 (*gray bars*) on induction medium agar (IMA) with different agar concentrations (2% or 5%) containing acetosyringone. Data are presented as the average number of hygromycin-B-resistant colonies from three independent experiments. *Error bars* indicate standard deviation

some compounds such as helicobasidin from *H. mompa* inhibit growth of *A. tumefaciens* during cocultivation.

Optimization of transformation conditions

We determined the influence of the following two factors on the transformation efficiency: (1) the effect of various *A. tumefaciens* strains and (2) the effect of different agar concentrations. The transformation efficiency was compared using three different strains of *A. tumefaciens*: AGL-1, EHA105, and MAFF301222, which were used to transform mycelia from *H. mompa* strain V17. The ATMT experiments were repeated independently three times, and the average number of resistant colonies grown on the plate was counted (Fig. 3). There were differences in transformation efficiencies among the strains. AGL-1 was found to be the most effective strain. The transformation efficiency using AGL-1 was 2.4–2.7 times and 16.0–16.5 times higher than EHA105 and MAFF301222, respectively. MAFF301222 was not good for transformation. Various *Agrobacterium* strains have been used to transform *Cryphonectria parasitica*. In *C. parasitica*, *Agrobacterium* AGL-1 was more efficient for transformation than LBA4404 (Park and Kim 2004). These results suggest that the difference in transformation efficiency was due to the genetic background of the *A. tumefaciens* strains (Krishnamohan et al. 2001), the effect of inhibitor from *H. mompa*, and the strain specificity of *A. tumefaciens* for *H. mompa* strains.

In three independent experiments using IMA medium with different agar concentrations, the results indicated that more hygromycin-B-resistant colonies were obtained in 5% agar than in 2% agar (Fig. 3). Although IMA with 1.5–2% agar is most commonly used for cocultivation of *Agrobacterium* and filamentous fungi for ATMT (Michielse et al. 2005), our results with *H. mompa* indicate that 2% agar gave lower efficiency of transformation than 5% agar. Based on these results, it is possible that the firmness of the agar may affect the distribution of the *Agrobacterium* cells and mycelia or inhibit their interaction (Zeilinger 2004). Moreover, the mycelial growth rate of *H. mompa* on IMA with 5% agar was faster than 2% agar, thereby resulting in differences in transformation efficiency.

Analysis of transformants

Putative hygromycin-B-resistant strains of *H. mompa* V17 were analyzed by PCR and Southern blot analysis to determine insertion of the resistance gene after transformation. In the PCR analysis (using hph1 and hph2), the isolated resistant strains showed a 626-bp-amplified fragment of the *hph* gene (Fig. 4a), whereas no amplified fragments appeared using a template derived from wild-type strain V17. When sequenced, the amplified DNA bands corresponded to the expected 626-bp internal fragment of the *hph* gene (data not shown).

To determine the T-DNA copy number, Southern blot hybridization was performed with genomic DNA extracted from the putative hygromycin-B-resistant and wild-type strain V17. The resistant strains contained a single copy of T-DNA in their genomes, with no signals at any position in the wild-type strain V17 (Fig. 4b). Southern blot analysis of strain V17 showed hybridizing band, indicating that the T-DNA was integrated at chromosomal locations. Some

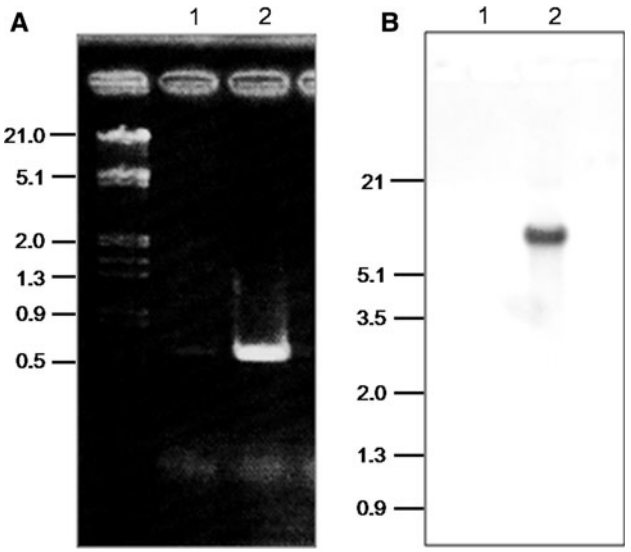

Fig. 4 **a** Polymerase chain reaction (PCR) and **b** Southern blot analysis of the *hph* gene from putative hygromycin-B-resistant colonies of *Helicobasidium mompa*. **a** PCR amplification was carried out using genomic DNA and primers hph1 and hph2, amplifying an approximately 0.6-kb fragment. *M*, DNA molecular-size markers (in kilobases) are indicated on the *left*; *lane 1* wild-type strain V17; *lane 2* transformant V17-TA25. **b** Genomic DNA (1 μg) was digested with *Hin*dIII and separated on 0.8% agarose gels. Hybridization was performed at 72°C using a digoxigenin (DIG)-labeled *hph* gene probe. *Lane 1* wild-type strain V17; *lane 2* transformant V17-TA25. The positions of molecular DNA size markers (in kilobases) are shown on the *left*

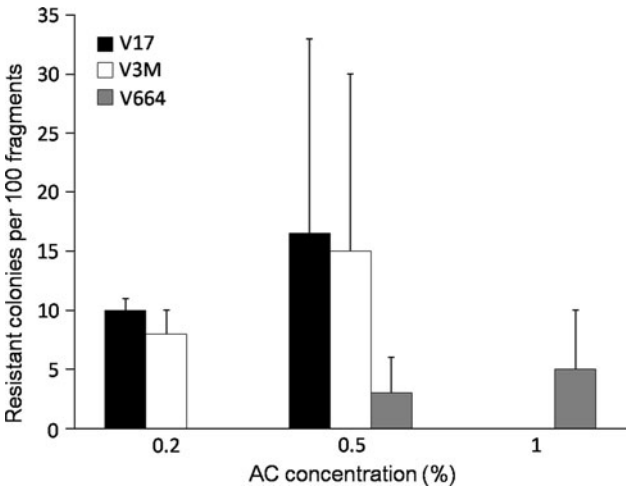

Fig. 5 The effect of activated carbon (AC) in induction medium agar (IMA) on transformation efficiency of *Helicobasidium mompa*. Three *H. mompa* strains (V17, V3M, and V664) were cocultivated with *Agrobacterium tumefaciens* strain EHA105 on IMA (2% agar) with acetosyringone. Data are presented as the average number of hygromycin-B-resistant colonies from three independent experiments. *Error bars* indicate standard deviation

studies have reported that multiple integrations predominate in T-DNA transfer to filamentous fungi (Micosh et al. 2001). However, similar results to those obtained in our analysis have been reported in *Clitopilus passeckerianus* (Kilaru et al. 2009) and *Venturia inaequalis* (Fitzgerald et al. 2003), where a majority of transformants contained a single copy of T-DNA in the genome. Therefore, the genetic manipulation of *H. mompa* may be possible because *A. tumefaciens* transformation resulted in single-copy integration.

Effect of AC concentration on transformation efficiency

Transformation efficiency was compared using different concentrations of AC during cocultivation. ATMT experiments using three *H. mompa* strains (V17, V3M, and V664) were repeated independently three times, and the average number of hygromycin-B-resistant colonies grown on SM was counted (Fig. 5). For V17 and V3M, which have little influence on the growth of *A. tumefaciens*, the highest numbers of resistant colonies were obtained when IMA with 0.5% AC was used, whereas no resistant colonies were observed in cocultivation using 1% AC. Interestingly, for V664, which has a high influence on the growth of *A. tumefaciens*, more resistant colonies were obtained in cocultivation using 1% AC, although we could not obtain any colonies when IMA with 0.2% AC was used. These results suggest that ATMT of *H. mompa* is hampered due to both some inhibitors and the high background growth of *H. mompa* during cocultivation without AC and that the transformation efficiency was increased using the AC, because the AC in IMA could adsorb the inhibitors from *H. mompa* and reduce high background growth of *H. mompa*. To reduce background growth, Zeilinger (2004) and Leclerque et al. (2004) described an overlay technique for *Trichoderma atroviride* and a harvesting technique for *Beauveria bassiana*. For these reasons, AC is necessary for ATMT using *H. mompa* strains that influence the growth of *A. tumefaciens* in order to reduce background growth. However, the transformation efficiency was decreased using 1% AC for V17 and V3M strains compared with 0.2% or 0.5%. This result suggests that the AC may adsorb not only some inhibitors but the acetosyringone that induces the virulence gene of *A. tumefaciens*.

It has been reported that ATMT is useful and more efficient for fungal transformation than a protoplast-based transformation system (Michielse et al. 2005). There are a number of factors influencing ATMT efficiency in fungi, for example, *A. tumefaciens* strain, cocultivation conditions, and acetosyringone concentration. However, there is little information as to what factors affect the efficiency of ATMT. In this study, ATMT was optimized in cocultivation with *H. mompa* by using different *Agrobacterium* strains, agar concentrations, and amounts of AC. ATMT,

which does not require protoplasts, is suitable for transformation of *H. mompa*. This is the first report of the successful transformation of *H. mompa*. The ATMT system for *H. mompa* will greatly facilitate molecular analysis of this important fungus.

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