Phytopathology • 2019 • 109:1404-1416 • https://doi.org/10.1094/PHYTO-01-19-0013-R

Mycology

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Green Fluorescent Protein Transformation Sheds More Light on a Widespread Mycoparasitic Interaction

Márk Z. Németh,[1] Alexandra Pintye,[1] Áron N. Horváth,[1] Pál Vági,[1,2] Gábor M. Kovács,[1,2] Markus Gorfer,[3] and Levente Kiss[1,4,†]

[1] Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, H-1525 Budapest, Hungary
[2] Department of Plant Anatomy, Institute of Biology, Eötvös Loránd University, H-1117 Budapest, Hungary
[3] Austrian Institute of Technology, BOKU University of Natural Resources and Life Sciences, A-3430 Tulln, Austria
[4] Institute for Life Sciences and the Environment, Centre for Crop Health, University of Southern Queensland, Toowoomba, Queensland 4350, Australia

Accepted for publication 18 March 2019.

ABSTRACT

Powdery mildews, ubiquitous obligate biotrophic plant pathogens, are often attacked in the field by mycoparasitic fungi belonging to the genus *Ampelomyces*. Some *Ampelomyces* strains are commercialized biocontrol agents of crop pathogenic powdery mildews. Using *Agrobacterium tumefaciens*-mediated transformation (ATMT), we produced stable *Ampelomyces* transformants that constitutively expressed green fluorescent protein (GFP) to (i) improve the visualization of the mildew–*Ampelomyces* interaction and (ii) decipher the environmental fate of *Ampelomyces* fungi before and after acting as a mycoparasite. Detection of *Ampelomyces* structures, and especially hyphae, was greatly enhanced when diverse powdery mildew, leaf, and soil samples containing GFP transformants were examined with fluorescence microscopy compared with brightfield and differential interference contrast optics. We showed for the first time, to our knowledge, that *Ampelomyces* strains can persist up to 21 days on mildew-free host plant surfaces, where they can attack powdery mildew structures as soon as these appear after this period. As saprobes in decomposing, powdery mildew-infected leaves on the ground and also in autoclaved soil, *Ampelomyces* strains developed new hyphae but did not sporulate. These results indicate that *Ampelomyces* strains occupy a niche in the phyllosphere where they act primarily as mycoparasites of powdery mildews. Our work has established a framework for a molecular genetic toolbox for the genus *Ampelomyces* using ATMT.

Keyword: tritrophic interactions

Mycoparasites (i.e., fungi that parasitize other fungi) are commonly found in most terrestrial ecosystems, the best known species being those that attack fungal plant pathogens (Boddy 2015; Jeffries and Young 1994; Kiss 2001; Viterbo and Horwitz 2010). A number of mycoparasites have been long studied and commercially utilized as biocontrol agents (BCAs) of crop pathogens (Boddy 2015; Viterbo and Horwitz 2010). Others have been in focus as components of natural multitrophic relationships (Kiss 2001; Liyanage et al. 2018; Parratt and Laine 2016, 2018; Parratt et al. 2017; Tollenaere et al. 2014). Direct observation of interfungal parasitic relationships is notoriously difficult at the cellular or hyphal level, using classical light, scanning, and transmission electron microscopy (LM, SEM, and TEM, respectively) and other visualization methods (Calonje et al. 2000; Jacobs et al. 2005; Jeffries and Young 1994; Kim and Vujanovic 2018; Smith et al. 2008). Recombinant DNA technologies, such as those leading to the production of transformed strains that constitutively or inducibly express fluorescent proteins (e.g., green fluorescent protein [GFP]), have been applied in the study of mycoparasitism since these technologies became available in fungal biology research (Lorang et al. 2001). For example, experiments using GFP-expressing BCAs, such as *Clonostachys* and *Trichoderma* strains, resulted in improved visualization of mycoparasitic interactions between these fungi and their plant pathogenic mycohosts (Lu et al. 2004; Lübeck et al. 2002; Sarrocco et al. 2006). Bitsadze et al. (2015) visualized the degradation of *Sclerotinia sclerotiorum* sclerotia separately, and also simultaneously, by two transformed BCAs, *Microsphaeropsis ochracea* and *Paraphaeosphaeria minitans*, expressing red fluorescent protein (DsRed) and GFP, respectively. The attack of the cultivated mushroom *Agaricus bisporus* by *Lecanicillium fungicola* has also been studied with GFP-expressing mycoparasitic strains (Amey et al. 2002).

A remarkable example of a widespread natural interfungal parasitic relationship is the interaction between powdery mildews, common obligate biotrophic plant pathogens of more than 10,000 angiosperms, including important crops (Braun and Cook 2012; Glawe 2008), and pycnidial fungi belonging to the genus *Ampelomyces* (Kiss 2008). To our knowledge, this intricate interaction has not been examined previously by fluorescent protein biotechnology. In fact, this is the oldest known interfungal parasitic relationship, first revealed by De Bary in the nineteenth century using simple LM (Kiss 2008; Kiss et al. 2004), and later confirmed by detailed LM, SEM, and TEM studies (Hashioka and Nakai 1980; Kiss et al. 2010; Speer 1979; Sundheim and Krekling 1982). As De Bary (1870) and subsequent studies have shown, one-celled *Ampelomyces* conidia are released from pycnidia produced inside the powdery mildew mycelium by the rupture of the pycnidial wall; conidia then germinate on the host plant surfaces, penetrate powdery mildew hyphae found in their vicinity, and invade these

†Corresponding author: L. Kiss; Levente.Kiss@usq.edu.au

Funding: This project was supported by the Széchenyi 2020 Programme, the European Regional Development Fund, and the Hungarian Government (grant GINOP-2.3.2-15-2016-00061). The University of Southern Queensland, the Hungarian Research, Development and Innovation Office (grant NKFIH NN100415), and the Austrian-Hungarian Action Foundation (grant 90öu16) also supported a part of this work. M. Gorfer received additional support from the European Union FP7 ERA-Net CORE Organic II (project Vineman). A. Pintye's contribution was supported by a János Bolyai Research Scholarship from the Hungarian Academy of Sciences.

*The *e*-**X**tra logo stands for "electronic extra" and indicates that two supplementary figures and four supplementary tables are published online.

The author(s) declare no conflict of interest.

internally, growing from cell to cell through the septal pores of the mycohost (Hashioka and Nakai 1980; Speer 1979). The early stage of mycoparasitism is biotrophic (Kiss et al. 2010), but the invaded hyphal cells later begin to die, and a necrotrophic interaction results (Hashioka and Nakai 1980). This process ends with intracellular sporulation of *Ampelomyces* fungi—that is, production of pycnidia mostly inside powdery mildew conidiophores, immature conidia, and, if present, also in immature sexual fruiting bodies (chasmothecia) of powdery mildews (Falk et al. 1995a, b; Hashioka and Nakai 1980; Kiss et al. 2010).

Parasitized powdery mildew colonies continue their growth on host plant surfaces, but their asexual and sexual sporulation is reduced, or completely stopped, by *Ampelomyces* mycoparasites (Falk et al. 1995a, b; Legler et al. 2016; Romero et al. 2003; Shishkoff and McGrath 2002). A transcriptomic study revealed the upregulation of some genes related to toxin biosynthesis, together with other potentially mycoparasitism-related proteins such as secreted proteases and putative virulence factors during mycoparasitism (Siozios et al. 2015). However, approximately 50% of the *Ampelomyces* transcripts did not relate to any known protein sequences (Siozios et al. 2015). This may indicate that a part of the *Ampelomyces* proteome is unique, or this type of mycoparasitism has not been studied in sufficient detail in other interfungal parasitic relationships.

The natural occurrence of *Ampelomyces* fungi has been globally reported in dozens of powdery mildew species (Falk et al. 1995a; Kiss 1998; Liyanage et al. 2018). Phylogenetic analyses have revealed that *Ampelomyces* strains isolated from diverse powdery mildew species, and cultured on agar media, belong to distinct lineages that are not clearly associated with their original mycohosts (Angeli et al. 2012; Kiss et al. 2011; Liang et al. 2007; Liyanage et al. 2018; Park et al. 2010; Pintye et al. 2012, 2015; Szentiványi et al. 2005). All *Ampelomyces* strains tested were able to parasitize several powdery mildew species, in addition to their original mycohosts, both in the laboratory (Kiss et al. 2010; Legler et al. 2016; Liang et al. 2007; Romero et al. 2003; Shishkoff and McGrath 2002; Szentiványi et al. 2005) and field experiments (Kiss et al. 2011; Legler et al. 2016; Sztejnberg et al. 1989), after being subcultured on agar media. A formal taxonomic revision of the genus is still warranted (Legler et al. 2016). Powdery mildew species are each specialized to one or a few host plant species (Braun and Cook 2012; Glawe 2008), whereas genetically distinct *Ampelomyces* strains seem to be their generalist mycoparasites (Kiss et al. 2011; Legler et al. 2016; Pintye et al. 2012, 2015). These interactions are interesting natural tritrophic relationships (Kiss 2001, 2008) and have been included in a number of ecological studies (Parratt and Laine 2016, 2018; Parratt et al. 2017; Tollenaere et al. 2014). Because powdery mildews are themselves parasites, *Ampelomyces* strains are also called hyperparasites (Kiss 2001, 2008; Parratt and Laine 2016, 2018; Parratt et al. 2017). A number of *Ampelomyces* strains have been exploited and commercialized as BCAs of powdery mildews infecting grapes and vegetables (Kiss et al. 2004; Legler et al. 2016).

In the absence of mycohosts (i.e., living powdery mildew colonies), the activity of *Ampelomyces* strains in the field is an interesting and debated question. Brimner and Boland (2003) suggested that the application of *Ampelomyces* strains as a BCA may result in unwanted interactions with soil fungi, as nontarget effects of biocontrol procedures, although there is no evidence for such interactions (Kiss 2004). It has been repeatedly shown that several strains identified as *Ampelomyces* strains were confused with *Phoma*-like pycnidial fungi isolated from diverse environments (Aveskamp et al. 2010; Sullivan and White 2000; Szentiványi et al. 2005), which led to misconceptions about the biology of *Ampelomyces* (Aveskamp et al. 2010; Kiss 2008; Kiss et al. 2004). The only experimental evidence for survival of *Ampelomyces* fungi in the environment, in the absence of living powdery mildew colonies, was the detection of a number of overwintering strategies linked to powdery mildew structures in temperate regions (Falk et al. 1995a; Kiss et al. 2011; Szentiványi and Kiss 2003). Because *Ampelomyces* strains can be cultured on artificial media (Falk et al. 1995a, b; Legler et al. 2016; Liang et al. 2007; Liyanage et al. 2018; Park et al. 2010; Pintye et al. 2012, 2015; Sztejnberg et al. 1989), the possibility that these fungi persist in the environment as saprobes cannot be excluded. It is not known whether these mycoparasites develop further as saprobes in senescent leaves that were once infected with *Ampelomyces*-parasitized powdery mildews, as hypothesized by De Bary (1870) and Yarwood (1939) several decades ago, or whether *Ampelomyces* mycoparasites interact with soilborne fungi after those leaves have been decomposed at the soil level.

It is also not known what happens to *Ampelomyces* conidia on powdery mildew-free aerial plant surfaces after being either naturally released and splash dispersed from pycnidia produced in already parasitized powdery mildew colonies or sprayed on crops as BCAs. Clearly, a method to better visualize *Ampelomyces* structures inside and outside powdery mildew colonies, such as the use of GFP-expressing mutants in laboratory experiments, would greatly enhance the study of the environmental fate of *Ampelomyces* fungi in the absence of their mycohosts (i.e., before, after, or instead of acting as a mycoparasite) and would also shed more light on their development inside powdery mildew colonies. The objectives of this work were as follows: (i) transform *Ampelomyces* strains to produce mutants that constitutively express GFP in culture; (ii) characterize transformants in terms of transgene copy numbers; (iii) characterize transformants in terms of morphology and growth in culture as well as mycoparasitic activity compared with the wild-type strains; (iv) test whether GFP expression of selected transformants is stable during mycoparasitic development inside powdery mildew structures; (v) compare the visualization of the mycoparasitic interaction with GFP expression, using fluorescence microscopy, versus other optical microscopy techniques; (vi) determine whether *Ampelomyces* conidia can survive for at least 2 to 3 weeks on powdery mildew-free host plant surfaces; (vii) observe the fate of *Ampelomyces* structures during and after senescence of leaves infected with *Ampelomyces*-parasitized powdery mildew; and (viii) examine whether there is any saprotrophic development of *Ampelomyces* strains in autoclaved soil.

MATERIALS AND METHODS

Fungal and plant materials. A comprehensive screening compared the mycoparasitic activity of 33 *Ampelomyces* strains in grape powdery mildew (*Erysiphe necator*) as well as their growth and sporulation rate in culture (Legler et al. 2016). Among those 33 strains, two (RS1-a and GYER) were used in this study. RS1-a was originally isolated from *Podosphaera pannosa* infecting rose (*Rosa* sp.) and GYER from *Erysiphe arcuata* infecting hornbeam (*Carpinus betulus*). RS1-a was included in this study because it was the best performing strain in terms of mycoparasitic activity in grape powdery mildew and sporulation in culture (Legler et al. 2016), whereas GYER was selected for this work to test the transformation protocol on another distinct *Ampelomyces* genotype as well. The two stains were maintained on Czapek-Dox medium supplemented with 2% malt (MCzA) as described previously (Legler et al. 2016).

Mycoparasitic tests were performed with the following five powdery mildew species, maintained in the greenhouse on their respective host plants, grown in pots: *Erysiphe necator* infecting grapevine (*Vitis vinifera* 'Chardonnay'), *Podosphaera xanthii* infecting cucumber (*Cucumis sativus* 'Rajnai Fürtös'), *Blumeria graminis* f. sp. *hordei* infecting barley (*Hordeum vulgare* MW08-16), *Pseudoidium neolycopersici* infecting tomato (*Solanum lycopersicum* 'Kecskeméti Jubileum'), and *Leveillula taurica* infecting pepper (*Capsicum annuum* 'Total'). Potted grapevine

plants continuously produced young leaves that supported the maintenance of *Erysiphe necator* in the greenhouse throughout the season. Potted cucumber, barley, tomato, and pepper plants, infected with their respective powdery mildews, had to be replaced with newly grown and freshly inoculated plants every 3 to 6 weeks to ensure the continuous maintenance of the other four powdery mildew species in the greenhouse.

Phylogenetic analyses. An approximately 850-bp-long part of the actin gene (*act1*) of GYER was amplified and sequenced as described previously (Pintye et al. 2012) and deposited in GenBank under accession number MH879022. This sequence was aligned with the *act1* sequence data set used by Pintye et al. (2012) using MAFFT online (Katoh and Standley 2013) with the FFT-NS-i algorithm. The rDNA internal transcribed spacer (ITS) sequence data set used by Pintye et al. (2012) was supplemented with the ITS sequence of GYER, determined earlier (Kiss et al. 2011) and was aligned with MAFFT online using E-INS-i. These alignments were combined using MEGA6 (Tamura et al. 2013) to produce a concatenated ITS_act1 data set. The final alignment (TreeBase study ID 23290) was 1,333 characters long, consisting of 502 characters for the ITS and 831 for the *act1* data set. This combined alignment was introduced to RaxML raxmlGUI 1.5 (Silvestro and Michalak 2012; Stamatakis 2014) and a maximum likelihood analysis was conducted using the GTR+G substitution model and maximum likelihood estimation of base frequencies. Partitions were set to correspond to the two loci. Supports of the branches were calculated from 1,000 bootstrap replicates. The tree with the highest likelihood value was visualized in MEGA6 (Tamura et al. 2013) and TreeGraph 2.13.0 (Stöver and Müller 2010). *Phoma herbarum* CBS 567.63 was used as the outgroup based on a previous work (Pintye et al. 2012).

Transformation of *Ampelomyces* strains. Because hygromycin B (Sigma-Aldrich) was used as the selection agent during transformations, first, as a preliminary step, we determined the lowest concentration that causes complete growth inhibition of strains RS1-a and GYER in culture. The following concentrations of hygromycin B added to malt extract agar (MEA; Merck) were tested: 5, 10, 25, 50, and 100 mg/liter. Each concentration was tested by inoculating three plates with a 5-mm-diameter coeval mycelial disc of one of the two strains. These tests, performed twice, revealed that 50 mg/liter of hygromycin B completely inhibited the growth of both strains. Therefore, this concentration was used in the subsequent work.

Transformation of the two strains was performed based on previously published protocols (Gorfer et al. 2007; Hanif et al. 2002) as follows. First, 15 to 20 small fungal colony fragments, approximately 2 to 4 × 2 to 4 mm, were transferred to sterile cellophane sheets placed on MEA in 9-cm-diameter plates. Five such plates were prepared for RS1-a, and another five for GYER. Strains were grown for 4 days in dark at 23°C, then the cellophane sheets bearing the growing fungal colonies were transferred to plates containing Moser induction medium (MoserIND) (Gorfer et al. 2007).

Rhizobium radiobacter (previously known as *Agrobacterium tumefaciens*) strain AGL1 (Lazo et al. 1991) carrying the binary vector pCBCT (Gorfer et al. 2007) was used in the transformation work. This vector contains the *SGFP* gene with the *toxA* promoter (Lorang et al. 2001) and the *hph* gene driven by the *trpC* promoter (Carroll et al. 1994). Both promoters provide a stable constitutive expression of the respective genes (Lorang et al. 2001).

Bacteria were grown overnight at 28°C in 50-ml plastic tubes under continuous agitation (180 rpm) in a shaker in 4 ml of tryptic soy broth supplemented with 50 µg/ml of kanamycin. Bacteria were then pelleted by centrifugation for 9 min at 3,800 × *g* and resuspended in *Agrobacterium* induction medium (AtIND) (Gorfer et al. 2007). Their induction was reached during growth for 6 h under continuous agitation at 180 rpm at 28°C. Aliquots of the induced bacterial culture were pipetted directly on *Ampelomyces* colonies growing on cellophane sheets placed on MoserIND. Coculture plates were incubated for 4 days at room temperature, then cellophane sheets from these plates were transferred to 9-cm-diameter plates containing 25 to 28 ml of selective medium each (MEA with 50 mg/liter of hygromycin B and 100 mg/liter of cefotaxime; Duchefa Biochemie) and incubated in the dark at 22°C for 4 to 8 weeks, until emergence of visible fungal colonies. Hyphae and, if produced, conidia of actively growing putative transformants were illuminated with blue light and colonies exhibiting green fluorescence were transferred each to a new 6-cm-diameter plate with selective medium. Colonies exhibiting fluorescence when excited with blue light were considered as GFP transformants of the respective *Ampelomyces* strains. To produce monoconidial transformants, 1 ml of sterile water with 50 mg of chloramphenicol was pipetted onto each plate containing a sporulating colony of an RS1-a transformant and the colony surface was rubbed with a sterile artist's brush to release as many conidia as possible from pycnidia. Conidial suspensions, diluted 10^4 to 10^6 times with sterile water, were spread on new 6-cm-diameter plates containing MCzA. A similar procedure was carried out with the actively growing colonies of GYER transformants; suspensions produced from nonsporulating GYER colonies were diluted 100×, and hyphal fragments of these suspensions became the colony forming units. Plates were incubated for 1 week at room temperature, then the emerged individual colonies were transferred each to a new plate with MCzA and subcultured on the same medium 3 weeks later. GFP expression of hyphae and, if produced, conidia of the subcultured colonies was verified with a fluorescence microscope. These all exhibited green fluorescence under blue light (Fig. 1), and thus were considered as GFP transformants of the wild-type strains RS1-a and GYER, respectively. Seven transformants of RS1-a and six transformants of GYER were used in subsequent work; their designations are shown in Supplementary Table S1. These 13 transformants were subcultured every 4 to 6 weeks in 6-cm-diameter plates on MCzA. GFP expression of hyphae was verified with fluorescent microscopy before every subculturing. Hygromycin B was not added to MCzA used to maintain the transformants in culture because these remained stable without selection pressure during the duration of this work.

Polymerase chain reaction verification of transfer DNA insertion into transformants. DNA was extracted from the 13 transformants as described earlier (Cubero et al. 1999), with centrifugation lengths extended to 20 min. Insertion of transfer DNA (T-DNA) in the transformants' genomes was checked by amplification of two fragments of T-DNA, *hph* and *SGFP*, using primers hph-F1/hph-qR2 and GFPF/GFPR (Sarrocco et al. 2006). Primers hph-F1 and hph-qR2 were designed based on the pCBCT sequence (Gorfer et al. 2007) using SnapGene Viewer software (version 4; GSL Biotech). In these amplifications, Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) was used according to the manufacturer's recommendations. The polymerase chain reaction (PCR) program was as follows: 98°C denaturation for 2 min, followed by 35 cycles each consisting of 98°C for 10 s, 57°C (for *hph*) or 66°C (for *SGFP*) for 10 s, and 72°C for 30 s, and concluded with a final extension at 72°C for 5 min. Negative controls (ultrapure water) and nontemplate controls (DNA from the respective wild-type strains) were included in all amplifications. The resulting PCR products were separated on 1% agarose gel and visualized using GelRed Nucleic Acid Gel Stain (Biotium) under ultraviolet illumination in the GelDoc-It system (UVP). Amplicons were sent for sequencing to LGC Genomics GmbH. Sequencing was done with primers hph-F1 and GFPF, respectively. Resulting sequences were aligned using MEGA7 (Kumar et al. 2016) using default settings.

Determination of T-DNA insert copy numbers. The copy number of T-DNA in transformants was determined by quantitative real-time PCR (qPCR) using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen 2001), the most robust

method for this purpose (Bubner and Baldwin 2004). This approach has already been shown to work well in fungal transformants (Solomon et al. 2008) and is based on the relative quantification of copy numbers of an unknown target region compared with a known copy number internal reference gene. We targeted *hph* of T-DNA as the unknown copy number region. A putatively single-copy gene, *euknr*, the eukaryotic nitrate reductase encoding gene (Gorfer et al. 2011), was sequenced in wild-type RS1-a and GYER strains with the Ascomycetes-specific nested PCR method described previously (Gorfer et al. 2011). The newly obtained sequences were deposited in GenBank under accession numbers MH879020 and MH879021. A BLAST search with these sequences on the publicly available *Ampelomyces* genome (https://genome.jgi.doe.gov/Ampqui1/Ampqui1.home.html) using the default settings of the webpage resulted in a single hit, indicating that *euknr* is a single-copy gene in these fungi. Thus, *euknr* was used as a single-copy internal reference gene in qPCR measurements.

To produce a calibrator sample for the $2^{-\Delta\Delta Ct}$ method, the *hph* gene of pCBCT and an approximately 1-kb fragment of *euknr* of RS1-a and GYER were amplified with Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher Scientific). Primers trpCP02/CB02 (Gorfer et al. 2007) were used to amplify *hph* and niaD31F (for RS1-a) or niaD31F-alt (for GYER)/niaD32R to amplify *euknr*. Primers niaD31F, niaD31F-alt, and niaD32R were designed with SnapGene Viewer software using the *euknr* sequences determined earlier to allow direct amplification of *euknr* from our strains. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and the fragment containing *hph* was subjected to a treatment with the Fast DNA End Repair Kit (Thermo Fisher Scientific) to produce 5′ phosphorylated DNA for blunt end ligation. Resulting treated DNA was purified with the QIAquick PCR Purification Kit. Ligation was done with a Rapid DNA Ligation Kit (Thermo Fisher Scientific) and the resulting ligation mixture was used as a target for PCR with primers trpCP02/niaD32R to amplify the targeted *hph_euknr* fusion product. The product was purified from 1% agarose gel with the GenElute Gel Extraction Kit (Sigma-Aldrich) and cloned into pJET1.2/blunt vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). Resulting plasmid products were transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells (Invitrogen). One positive clone was selected for *hph* fused to *euknr* of RS1-a, and another one for *hph* fused to *euknr* of GYER. These clones were grown separately overnight in lysogeny broth medium and used for plasmid extraction with the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). PCR amplifications, product purifications, phosphorylation, ligation, cloning, and plasmid extraction were conducted according to the manufacturers' protocols. Plasmid solutions of approximately 5×10^5 to 10^6 copies/µl were produced and used in qPCR as 1:1 copy number calibrators.

For qPCR measurements, DNA was extracted from the 13 transformants using the DNeasy Plant Mini Kit (Qiagen). qPCR primers were designed manually with the aid of SnapGene Viewer software. Primers hph-qF2 and hph-qR2 were designed based on the pCBCT sequence (Gorfer et al. 2007), whereas primers qNR-F2/qNR-R2 were designed based on the *euknr* sequences of RS1-a and GYER determined in this study. First, these primer sets were tested during qPCRs using dilution series (undiluted DNA, and 2×, 5×, 10×, 50×, and 100× dilutions in ultrapure water) of RS1-a transformant A2 to determine efficiency and range of linearity. Reactions were run in triplicate in a Bio-Rad CFX96 Touch C1000 qPCR machine in 10-µl final volumes with 5 µl of iTaq Universal SYBR Green Supermix (Bio-Rad), 0.5 µl of each primer, and 1 µl of DNA extract, using the following PCR program: denaturation at 95°C for 5 min, followed by 30 cycles each consisting of 95°C for 10 s, 59°C for 10 s, and 72°C for 30 s. Specificity of the reactions was checked by registering a melt curve. A negative control reaction was included in each qPCR. Measured qPCR efficiencies were approximately 90 and 89%, respectively, and all of the tested dilutions were found to fit the linear range. Correlation coefficients (R^2) of regression curves were 0.992 for *hph* and 0.998 for *euknr* amplifications. The same qPCR protocol and reaction composition were used for copy number measurements using plasmid extracts and transformants' DNA samples as targets. Copy numbers were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Supplementary Table S2.

Morphology and saprobic growth characteristics of transformants. The morphology of the coeval colonies of the seven RS1-a and the six GYER transformants, grown on MCzA, as well as the morphology of their pycnidia and conidia produced in

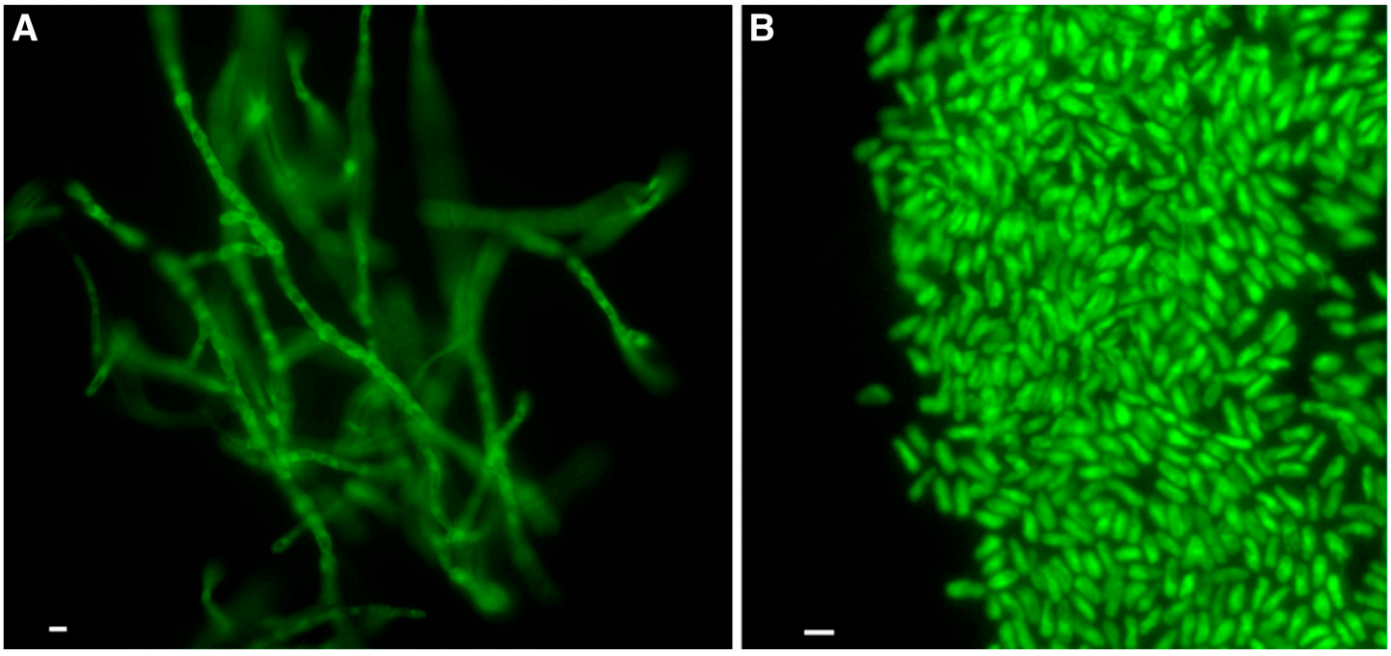

Fig. 1. Green fluorescence-emitting hyphae and conidia of transformant B3 of *Ampelomyces* strain RS1-a 2 weeks after its first transfer to a plate without the selection agent. **A,** Hyphae. **B,** Conidia. Bar = 5 µm in A and 10 µm in B.

culture and examined with differential interference contrast (DIC) optics, were compared with those of the wild-type RS1-a and GYER strains to observe whether any obvious morphological differences were developed as a result of the transformation. Saprotrophic growth of transformants was measured during growth tests at 22°C as described previously (Legler et al. 2016) with eight replicates of each transformant. High-resolution photos of 4-week-old colonies were used to measure the area of each fungal colony using ImageJ software version 1.51 (Schneider et al. 2012).

Setup of mycoparasitic tests with transformants. Because the sporulation of the wild-type GYER strain and its transformants has become unreliable during subculturing, gradually decreasing over time, sometimes it was impossible to harvest conidia from their cultures for powdery mildew inoculation experiments. Thus, only the results obtained with the wild-type RS1-a strain and its seven transformants are presented here. These experiments were performed to test whether mycoparasitic activity was altered in terms of penetration of powdery mildew hyphae, intrahyphal growth, and production of intracellular pycnidia, as a result of transformation. Young, sporulating *P. xanthii* colonies on potted cucumber plants were inoculated with conidial suspensions, 10^6 conidia/ml, prepared from each of the seven RS1-a transformants and the wild type as described earlier (Szentiványi et al. 2005). Conidial suspensions were sprayed each on three plants until runoff. To verify the viability/GFP expression of *Ampelomyces* conidia used in these treatments, 30 µl of each conidial suspension was spread immediately after inoculations in a 6-cm-diameter plate containing 1.5% water agar medium covered by one layer of sterile cellophane. Plates were incubated at room temperature for 24 h, then cellophane pieces bearing fungal material were cut out, placed on a slide, and examined with a fluorescence microscope as detailed below. Inoculated plants were kept in closed transparent isolation chambers for 10 days at 22°C, 80 to 90% relative humidity (RH), and 16 h of daily illumination. Three powdery mildew-infected plants sprayed with water until runoff and kept in another isolation chamber served as negative controls. Samples of the powdery mildew mycelia treated with *Ampelomyces* strains, or water in the case of negative controls, were microscopically examined as described below. The experiment was carried out three times: 4, 5, and then 30 months after production and repeated subculturing of the transformants.

Microscopy. Parts of the powdery mildew mycelia treated with *Ampelomyces* strains were removed from leaves with 3- to 5-cm-long pieces of crystal clear cellotape. These were placed on microscope slides, in droplets of distilled water or 10% glycerol, without being covered with coverslips, and first examined with brightfield, DIC, and/or phase contrast microscopy with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Microscopy GmbH). GFP fluorescence was detected in epifluorescent mode using a fluorescent filter set composed of a 450- to 490-nm excitation filter, a 495-nm dichroic mirror, and a 500- to 550-nm barrier filter. Photographs were taken with a Zeiss AxioCam ICc5 camera using Zeiss ZEN 2011 software. Adobe Photoshop was used for minor color and contrast corrections on microphotographs, without any content changes.

To prepare samples for confocal laser scanning microscopy (CLSM), one or more *Ampelomyces* intracellular pycnidia together with a small part of the parasitized powdery mildew mycelium were removed with glass needles from leaves under a dissecting microscope and placed on a microscope slide, in a droplet of water, to be first examined with brightfield microscopy. CLSM observations were performed using a Carl Zeiss 410 LSM as described previously (Vági et al. 2014). Two-micrometer-thick optical slices were recorded and 10 to 20 slices were used to compile projections.

Mycoparasitic activity of RS1-a transformants in *P. xanthii* colonies on cucumber. To compare mycoparasitic activities (i.e., penetration of powdery mildew hyphae, intrahyphal growth, and intracellular pycnidial development) of the seven RS1-a transformants to the wild-type strain in the experimental setup described above, powdery mildew colonies were sampled with cellotape 10 days after inoculations with *Ampelomyces* strains and examined with brightfield, DIC, and fluorescence microscopy, together with samples taken from powdery mildew-infected plants that served as negative controls. CLSM observations were also performed. At the same time, to quantify the levels of mycoparasitism, nine individual powdery mildew colonies were randomly selected on three cucumber leaves for each transformant and the wild-type strain, and the number of intracellular pycnidia produced in conidiophores of *P. xanthii* was determined under a stereomicroscope in each colony, in a 1.25-mm^2 area, using a method developed previously (Kiss et al. 2011).

Statistical analyses. Statistical analyses were conducted using IBM SPSS 16.0 software. All of the results were expressed as the mean ± standard deviation data of eight replicates. Data determined during colony growth tests in culture and quantification of mycoparasitic activities of transformants and the wild-type strain in *P. xanthii* on cucumber were checked for normality of the data and homogeneity of variances using the Kolmogorov-Smirnov test and Levene's test, respectively. The transformants' values were compared with those of the wild type used as the control in one-way analysis of variance (with an alpha value of 0.05) coupled with either the two-sided Dunnett or Dunettt T3 post hoc test. All of the analyses were run with 95% confidence intervals. Descriptive statistics and additional details of statistical analyses are given in Supplementary Table S3.

Mycoparasitic activities of transformants in other powdery mildew fungi. To check the effectiveness of GFP expression of RS1-a transformants in the visualization of mycoparasitic activity of *Ampelomyces* fungi in powdery mildews other than *P. xanthii* infecting cucumber, mycoparasitic tests were carried out with *Erysiphe necator* infecting grapevine, *B. graminis* infecting barley, *Pseudoidium neolycopersici* infecting tomato, and *L. taurica* infecting pepper according to the setup described above. Transformants used in these tests are listed in Supplementary Table S4. Each experiment was carried out at least twice. Brightfield, phase contrast, and DIC microscopy as well as CLSM were used to examine mycelial samples taken at the end of each experiment. One of the transformants, B3, was reisolated from *P. xanthii* colonies as described earlier (Liang et al. 2007) and checked for GFP fluorescence and hygromycin B resistance on selective medium.

Testing survival and potential development of *Ampelomyces* fungi on cucumber in the absence of the mycohost. To examine whether *Ampelomyces* fungi can survive for up to 3 weeks on powdery mildew-free host plant surfaces, conidial suspensions of the wild-type RS1-a strain and those of two transformants, C10 and G4, were prepared, and the viability/GFP expression of *Ampelomyces* conidia was checked on 1.5% water agar medium as described above. Conidial suspensions from these three *Ampelomyces* materials were each sprayed until runoff on the following: (i) 42 potted healthy cucumber seedlings with three true leaves each, produced in a powdery mildew-free growth chamber (these plants were infected with powdery mildew sequentially, 4 to 21 days after inoculation, in groups of six, as detailed below); (ii) six other healthy cucumber seedlings from the same plant cohort, to monitor the development of *Ampelomyces* fungi in the absence of powdery mildew for up to 21 days; and (iii) six older cucumber plants, with six to eight true leaves each, heavily infected with powdery mildew, to serve as positive controls. Six healthy seedlings from the cohort with three true leaves served as negative controls and were sprayed with water. Each plant was kept in isolation, under a transparent cover, at 25°C, 80 to 90% RH, and 16 h of daily illumination until the end of the experiment, which was carried out twice.

To examine whether *Ampelomyces* strains can parasitize powdery mildew colonies after being present on powdery mildew-free plant

surfaces for up to 3 weeks, at 4, 7, 10, 12, 14, 18, and 21 days after treatments with RS1-a, C10, G4, and water, respectively, six plants per treatment were infected with *P. xanthii* by dusting conidia onto their leaves from coeval powdery mildew colonies maintained on other potted plants in the greenhouse. The young powdery mildew mycelium developed on each plant was examined with brightfield, DIC, and fluorescence microscopy 10 days after infection for the presence of *Ampelomyces* structures, including GFP-expressing pycnidia on leaves treated with C10 and G4.

To monitor the fate of *Ampelomyces* fungi on powdery mildew-free cucumber seedlings, approximately 2- × 2-cm areas of the inoculated leaf surfaces, two per plant and treatment, were sampled 10, 14, and 21 days after treatments, with cellotape pieces gently applied and then removed from the treated leaves with a forceps. Cellotape pieces were examined with brightfield and fluorescence microscopy to detect germination and further development of *Ampelomyces* conidia.

The presence of intracellular pycnidia of RS1-a, C10, and G4 on the powdery mildew-infected plants used as positive controls was also examined with brightfield and fluorescence microscopy.

Testing survival and potential development of *Ampelomyces* fungi in senescent/decomposed powdery mildew-infected leaves. This experiment used those powdery mildew-infected cucumber leaves that were the positive controls in the survival tests described above (i.e., pycnidia of RS1-a, C10, and G4 were present in abundance in powdery mildew colonies at the end of the experiment). To model leaf fall, the powdery mildew-infected parts of the leaves, bearing *Ampelomyces* pycnidia, were cut into approximately 2-cm^2 pieces under a dissecting microscope; these were then placed on the soil in the respective pots that still contained the original plant. Pots continued to be kept in isolation and they were watered regularly, as described above. Every 3 to 6 days, two to four leaf pieces were removed from each pot and microscopically examined for the presence of *Ampelomyces* structures, including GFP-expressing pycnidia on leaves treated with C10 and G4, as detailed above. Together with the decomposing leaf tissue samples, 0.5 to 1 g of soil was also collected under and around the removed leaf pieces, suspended in water or 10% glycerol droplets on microscope slides, and examined under a microscope as described above. Mounting samples in water or glycerol was important because it was noted that the fluorescent signal was much weaker when samples were checked without hydration. The experiment was carried out twice.

Testing survival and potential development of *Ampelomyces* fungi in autoclaved soil. Glass plates, 9 cm in diameter, were filled with commercial gardening soil (10 g per plate), closed, and autoclaved twice. Conidial suspensions of the wild-type RS1-a and transformants B3 and C10 were prepared and checked for viability/GFP expression on 1.5% water agar medium as detailed above. Three plates with autoclaved gardening soil were inoculated each with 300 µl of conidial suspension of one of the fungal materials by pipetting 100 µl of inoculum three times into three spots of plates that were then sealed with Parafilm and incubated at 22°C in the dark for 30 days. Three plates served as negative controls, and each was treated with 300 µl of sterile water. The autoclaved soil remained wet in plates sealed with Parafilm until the end of the experiment. Approximately 0.5- to 1-g soil samples were collected at random in each plate from 10 places, 7 and then 30 days after the treatments, suspended in water or 10% glycerol droplets on microscope slides, and microscopically examined for the presence of *Ampelomyces* structures, including GFP-expressing structures in the case of treatments with B3 and C10, as described above. The experiment was carried out twice.

RESULTS

Production and molecular characterization of transformants. Two *Ampelomyces* strains, RS1-a and GYER, isolated from rose and hornbeam powdery mildews, *P. pannosa* and *E. arcuata*, respectively, were selected for this work based on previous data on their cultural characteristics and mycoparasitic activities (Legler et al. 2016). The two *Ampelomyces* strains are phylogenetically distantly related (Supplementary Fig. S1). As a result of the *Agrobacterium tumefaciens*-mediated transformation (ATMT) (Idnurm et al. 2017), hygromycin B-resistant colonies appeared 8 to 10 weeks after cocultivation. These all exhibited continuous, albeit slow, growth in these plates, similar to the growth rate of wild-type *Ampelomyces* strains (Legler et al. 2016; Liang et al. 2007). The transformation ratio was approximately 10% for RS1-a and 30% for GYER. Hyphae of all but one of the emerged colonies exhibited strong green fluorescence when excited with blue light (Fig. 1A); the colony that barely showed any fluorescence was excluded from further studies. Those colonies that sporulated on the selective medium produced fluorescent conidia (Fig. 1B). Colonies that were fluorescent under blue light were considered as putative GFP transformants of the respective *Ampelomyces* strain. Of these, seven were selected for RS1-a and six for GYER and included in subsequent works. Their colonies were subcultured every 4 to 6 weeks on MCzA without hygromycin B, the selection agent. Maintenance of hygromycin B resistance and GFP fluorescence in the absence of selection pressure throughout our 3-year project indicated high mitotic stability of the transgenes.

PCRs targeting the amplification of *hph* and *SGFP*, two fragments of T-DNA, resulted in products with expected sizes in all of the tested transformants. Amplicon sequences were identical to partial *hph* and *SGFP* fragments of pCBCT (Gorfer et al. 2007), the source of T-DNA. No products were amplified from the wild-type strains RS1-a and GYER during these PCRs. qPCR measurements of T-DNA copy numbers showed that eight of the selected transformants contained a single copy of the insert, four transformants had two, and one had three copies of the T-DNA.

Morphology and growth of transformants in culture. Neither morphology of coeval colonies of the seven monoconidial RS1-a transformants nor morphology of their pycnidia and conidia produced in culture differed from the characteristics of the wild-type RS1-a strain. In addition, colony morphology of the six GYER transformants was similar to that of the wild-type strain. Pycnidia were only sporadically produced by both the wild type and the transformant GYER colonies in culture; when this happened, no differences were observed in the morphology of pycnidia and conidia of the wild-type strain and the transformants. Colony growth analysis revealed significant differences in saprobic growth of four of six GYER ($P \leq 0.0001$) and one of seven RS1-a ($P = 0.004$) transformants compared with the growth of the respective wild-type strains (Fig. 2), but the overall colony growth patterns described for a number of genetically different *Ampelomyces* strains (Kiss 1997; Legler et al. 2016; Liang et al. 2007) were not markedly altered in any of the RS1-a or GYER transformants. Two transformants with significantly higher growth rates contained one, another had two, and the fourth had three copies of T-DNA, whereas the other transformants characterized by growth rates similar to the wild-type strain had one or two T-DNA inserts.

Mycoparasitic activities of transformants in mycelia of different powdery mildew species. All experiments targeting the mycoparasitic activities of transformants in powdery mildew colonies, compared with the wild-type strain, were carried out with RS1-a materials. The sporulation of the wild-type GYER strain and its transformants was unreliable in culture; therefore, conidial suspensions could not always be prepared for mycoparasitic tests with GYER materials.

The first mycoparasitic tests were carried out with cucumber plants infected with *P. xanthii*, a common powdery mildew species infecting a number of vegetables, ornamentals, and wild plants. Conidial suspensions of the seven RS1-a transformants, and the wild-type strain, were sprayed each on three powdery mildew-infected plants, and mycoparasitic interactions were examined after

a 10-day incubation period. Each treatment with *Ampelomyces* strains resulted in development of pycnidia inside conidiophores of *P. xanthii*. Hyphae and pycnidia of all transformants, produced intracellularly in powdery mildew colonies, similar to their conidia released from intracellular pycnidia, exhibited the GFP signal when examined with a fluorescence microscope (Fig. 3A to C). Green fluorescence was not observed when hyphae, pycnidia, and conidia of *Ampelomyces* strains were examined in samples taken from powdery mildew colonies inoculated with the wild-type RS1-a strain. *Ampelomyces* pycnidia were uniformly distributed in all powdery mildew colonies on all plants. Intracellular hyphae of the transformants that entered powdery mildew conidia were much better visualized with fluorescence microscopy compared with brightfield, phase contrast, and even DIC optics (Fig. 3D). Microscopic observations did not reveal any differences among mycoparasitic activities of the seven transformants and the wild-type strain in terms of penetration of powdery mildew hyphae, intrahyphal growth, intracellular pycnidial development inside powdery mildew conidiophores, and release of their conidia from intracellular pycnidia. No *Ampelomyces* structures were observed in samples taken from negative controls. The experiment was first carried out 4 then 5 months after the production of transformants in culture, and then for the third time with transformants that had already been subcultured for 30 months on MCzA, without hygromycin B, the selection agent. GFP expression of hyphae, pycnidia, and conidia of transformants, produced intracellularly in powdery mildew colonies, was as intensive in the third experiment as in the first two, which supported the stability of the GFP transformation in all seven RS1-a transformants. This was also shown by GFP signal emission and resistance to 50 mg/liter of hygromycin B of transformant B3 in culture, after being reisolated from a parasitized *P. xanthii* colony.

Mycoparasitic tests were done in the same way with *Pseudoidium neolycopersici* infecting tomato, *L. taurica* infecting pepper, *B. graminis* infecting barley, and *Erysiphe necator* infecting grapevine. Again, visualization of intracellular *Ampelomyces* structures, and especially the visualization of their hyphae inside the hyphae, conidiophores, and conidia of the parasitized powdery mildews, was much better with fluorescence microscopy compared with brightfield, phase contrast, and DIC optics (Fig. 3E to H). Furthermore, CLSM revealed that the cells of the walls of transformants' mature intracellular pycnidia did not exhibit a strong GFP signal (Fig. 4). When intracellular pycnidia were mature, with dark brown wall cells, it was mainly the green fluorescence of *Ampelomyces* conidia localized inside pycnidia that exhibited the GFP signal inside powdery mildew conidiophores under blue light. In some cases, this was also somewhat visible with fluorescence microscopy (Fig. 3B, C, and E), but it was only CLSM that clearly revealed the lack of strong GFP signal from the walls of transformants' pycnidia (Fig. 4).

Quantification of mycoparasitic activity of transformants in cucumber powdery mildew colonies. Mycoparasitic activity of the seven transformants and the wild-type strain was quantified based on the number of pycnidia produced in the conidiophores of *P. xanthii* on cucumber leaves, determined using an already established method (Kiss et al. 2011; Legler et al. 2016). No significant differences ($P > 0.1$) were found among the values determined for the individual treatments with the seven transformants compared with the wild type, the average number of intracellular pycnidia being 811 ± 66 pycnidia/cm^2 parasitized powdery mildew mycelium for transformants and 848 ± 178 pycnidia/cm^2 powdery mildew mycelium for the wild-type strain (Fig. 5).

Testing survival and potential development of *Ampelomyces* fungi on cucumber plants in the absence of the mycohost. To find out whether *Ampelomyces* conidia can survive for at least 2 to 3 weeks on powdery mildew-free host plant surfaces and, if so, whether such survived conidia can still initiate mycoparasitism of newly established powdery mildew colonies on the same host plant surfaces, two transformants, C10 and G4, and the wild-type RS1-a strain were sprayed each on powdery mildew-free cucumber plants. Fluorescent microscopy performed 10 days after treatments revealed that conidia of the transformants germinated and the emerged hyphae started to grow on the leaves

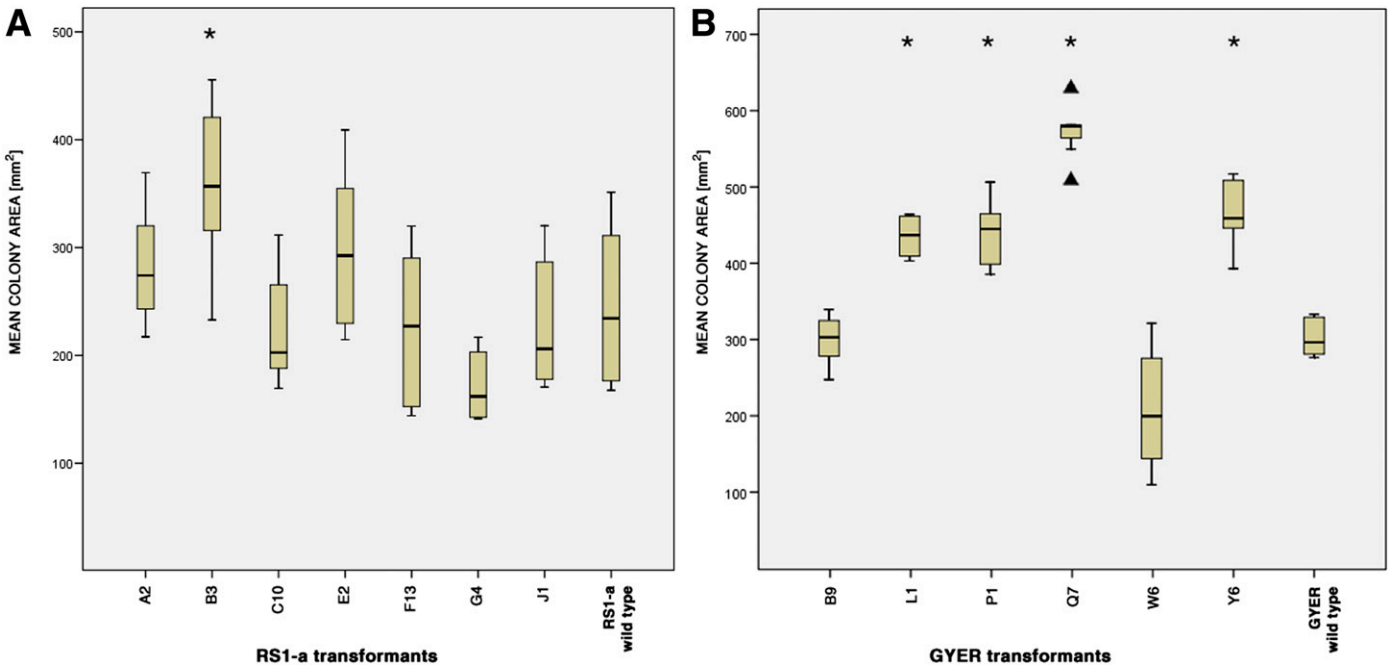

Fig. 2. Box plots showing surface area sizes of 4-week-old colonies of RS1-a and GYER transformants and the respective wild types. **A,** Seven RS1-a transformants and the wild-type strain. **B,** Six GYER transformants and the wild-type strain. Medians and second and third quartiles are shown as boxes; upper and lower whiskers represent the maximum and the minimum values of the data set, respectively. Transformants exhibiting growth that was significantly different from the wild type are marked with an asterisk (*$P < 0.05$). Triangles denote outliers.

(Supplementary Fig. S2). This was also detected with brightfield microscopy on plants inoculated with the wild-type strain. The visualization of the transformants' conidia with fluorescence microscopy was clearly superior to the detection of the germinated wild-type *Ampelomyces* conidia on leaf samples. No saprobic pycnidial production was observed on any leaves in the absence of powdery mildew.

Four, 7, 10, 12, 14, 18, and 21 days after being sprayed with one of the two transformants, or the wild-type strain, or water, six cucumber plants per treatment were infected with *P. xanthii*. Each infected plant cohort was examined 10 days after powdery mildew infection for the presence of *Ampelomyces* structures in the newly developed powdery mildew colonies. Mature intracellular pycnidia of *Ampelomyces* strains, releasing conidia, were detected in various numbers, and in small areas, in the 10-day-old powdery mildew colonies developed on all infected plants included in this experiment. Therefore, *Ampelomyces* mycoparasites survived for at least 3 weeks on powdery mildew-free cucumber plants and were able to penetrate and parasitize newly developed powdery mildew colonies even after a 21-day-long powdery mildew-free survival period. However, the level of mycoparasitism of powdery mildew colonies that had started to develop on leaves 10 to 21 days after *Ampelomyces* treatments was low, and the presence of *Ampelomyces* structures in powdery mildew hyphae and conidiophores was patchy, in contrast to what was seen in positive controls and also in previous experiments, where powdery mildew colonies had already covered large leaf areas when they were sprayed with *Ampelomyces* inoculum. GFP expression of transformants has greatly enhanced the study of the respective samples compared with those taken from powdery mildew colonies developed on leaves pretreated with the wild-type strain (Fig. 6).

Testing survival and potential development of *Ampelomyces* fungi in senescent/decomposed powdery mildew-infected leaves on the soil. *Ampelomyces* structures on powdery

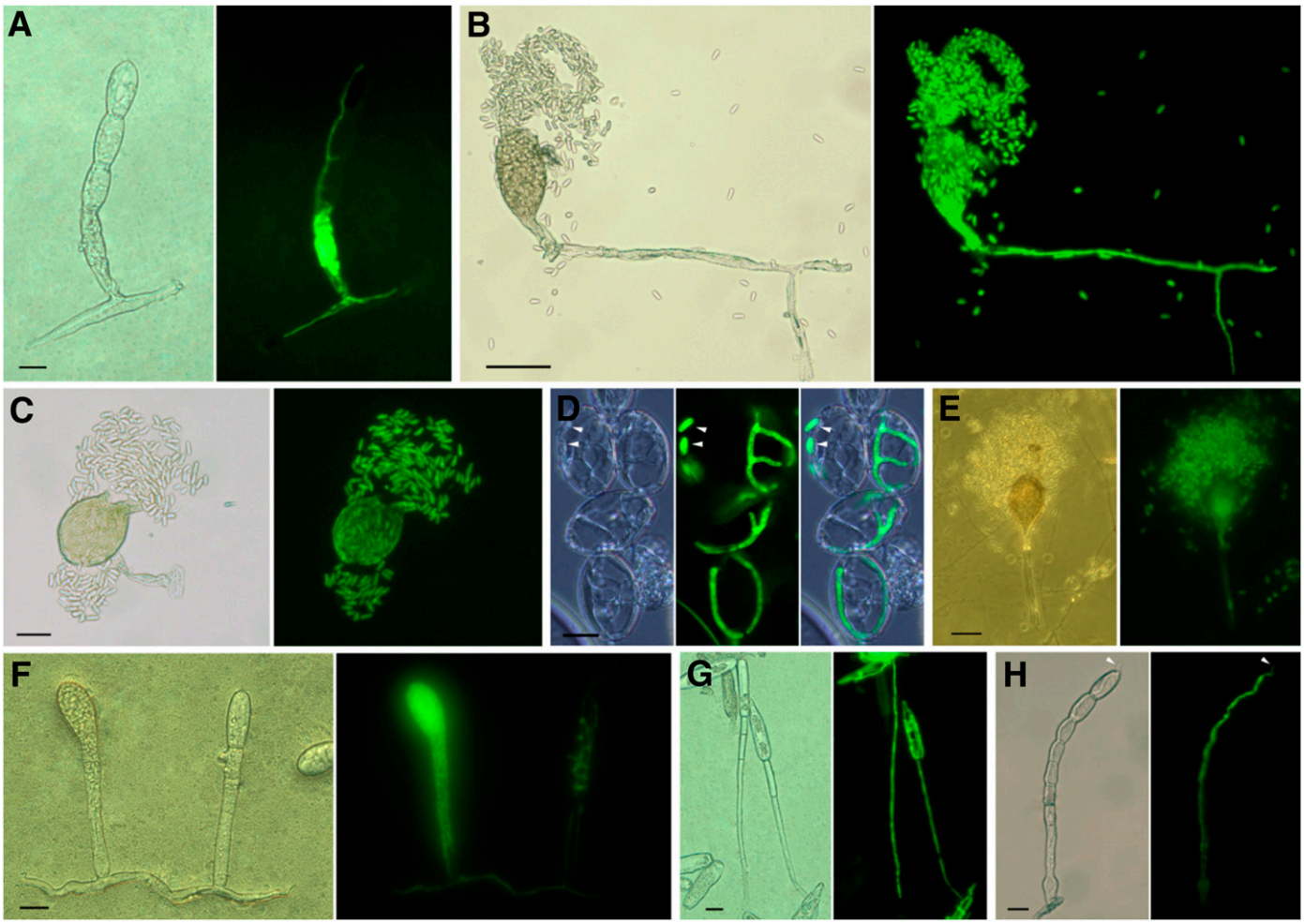

Fig. 3. Mycoparasitism of green fluorescent protein-expressing transformants of *Ampelomyces* strain RS1-a in five powdery mildew species, on their host plants, 10 days postinoculation. **A,** A young pycnidium of transformant J1 developing in the second cell of a conidiophore of *Podosphaera xanthii* on cucumber. Note also intracellular hyphae colonizing all of the other cells of the conidiophore. Left: micrograph with differential interference contrast (DIC) optics; right: fluorescence microscopy. **B,** A mature pycnidium of transformant G4 developed in a conidiophore of *P. xanthii* releasing conidia after rupture of the intracellular pycnidial wall. Note also intracellular hyphae in other parts of the mycelium. Left: DIC optics; right: fluorescence microscopy. **C,** A mature pycnidium of transformant B3 developed in *P. xanthii* and releasing conidia. Left: DIC optics; right: fluorescence microscopy. **D,** A close-up of hyphae of transformant G4 in conidia of *P. xanthii*. White arrows point to *Ampelomyces* conidia, outside of *P. xanthii* conidia. Left to right: DIC optics, fluorescence microscopy, and overlap of these two images. **E,** A pycnidium of transformant B3 developed in a conidiophore of *Erysiphe necator* on grape and releasing conidia. Left: bright field optics; right: fluorescence microscopy. **F,** Two conidiophores of *Pseudoidium neolycopersici* on tomato parasitized by transformant G4. A young pycnidium is developing in the conidiophore on the left; this process is even less advanced in the conidiophore on the right, where intracellular hyphae started to anastomose in the second and third cells. Note also intracellular hyphae in other parts of the mycelium. Left: DIC optics; right: fluorescence microscopy. **G,** Hyphae of transformant G4 growing inside conidiophores and conidia of *Leveillula taurica* on pepper. Left: DIC optics; right: fluorescence microscopy. **H,** Hyphae of transformant C10 growing inside a conidiophore of *Blumeria graminis* f. sp. *hordei* on barley. Note that the hypha grew out of the most distal powdery mildew conidium produced on this conidiophore (white arrow). Left: DIC optics; right: fluorescence microscopy. Micrographs in A, C, and E to H were taken from samples made with cellotape that reduced DIC effects but did not influence the quality of images taken with fluorescence microscopy. Bar = 20 µm in A, F, G, and H; 50 µm in B and C; and 10 µm in D and E.

mildew-infected leaf pieces placed on the soil in the respective plant's pots were detected for 30 days from the start of the experiment. Bright fluorescence of GFP transformants was observed during the first 9 to 12 days in the sampled and gradually decomposing leaf pieces; the fluorescent signal was always weaker in samples examined after this period. *Ampelomyces* pycnidia found on leaf pieces usually released conidia into the mounting medium during microscopic examinations, and not before, although the soil in pots was continuously kept wet. GFP fluorescence was not detected on leaf pieces inoculated with the wild-type strain, but background fluorescence of plant material and soil particles was always observed. Newly developed *Ampelomyces* hyphae were rarely seen in the senescent leaf tissues, whereas saprobic production of pycnidia in the decomposing leaves, and/or the adjacent soil samples collected from the pots, was not observed in this experiment.

Testing survival and potential development of *Ampelomyces* fungi in autoclaved soil. *Ampelomyces* conidia germinated and the emerging hyphae continued to grow in the sterile environment setup in this experiment. Hyphae of transformants were found on the surface of soil particles and also between particles as aerial hyphae. Wild-type hyphae were only detected as aerial hyphae, probably owing to difficulties in observing soil samples with brightfield, phase contrast, and DIC microscopy. GFP expression of transformants greatly enhanced the visualization of hyphae on and around soil particles (Fig. 7). *Ampelomyces* pycnidia were not detected in any plates with autoclaved soil in this 30-day-long experiment.

DISCUSSION

Heterologous fluorescent protein expression has been used in the study of mycoparasitic interactions, enhancing the visualization of interfungal parasitic relationships in diverse settings (Amey et al. 2002; Bitsadze et al. 2015; Lu et al. 2004; Lübeck et al. 2002; Sarrocco et al. 2006). This technology has proven to be extremely valuable in the study of the interactions between powdery mildews and *Ampelomyces* strains, for several reasons. First, the detection of *Ampelomyces* structures, and especially hyphae, was easier per se when diverse samples containing GFP transformants were examined with fluorescence microscopy compared with brightfield, phase contrast, and/or DIC observations of the same samples or samples containing wild-type *Ampelomyces* structures. Intracellular *Ampelomyces* hyphae have been identified with brightfield microscopy since the nineteenth century, first by De Bary (1870), and then also with other optics during their development inside powdery mildew hyphae, conidiophores, and conidia (Kiss et al. 2010; Speer 1979; Szentiványi and Kiss 2003). However, detection of *Ampelomyces* fungi could be difficult especially during the early stages of the mycoparasitic interaction, before the production of intracellular pycnidia in powdery mildew conidiophores. GFP expression of transformants greatly enhanced the visualization of their hyphae right after conidial germination on powdery mildew-free host plant surfaces, soon after their penetration into powdery mildew hyphae and also on the surface of soil particles, and distinguished these from hyphae of other fungi in experiments carried out in nonsterile environments.

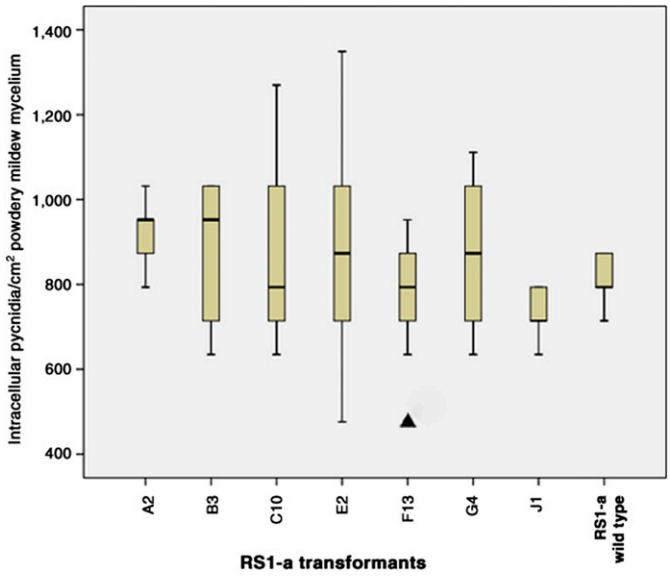

Fig. 5. Box plot of mycoparasitic activities of seven RS1-a transformants and the wild type expressed as the number of intracellular pycnidia developed in conidiophores of *Podosphaera xanthii* on cucumber per square centimeter of powdery mildew mycelium. Medians and second and third quartiles are shown as boxes; upper and lower whiskers represent the maximum and the minimum values of the data set, respectively. No significant differences from RS1-a wild type were detected ($P > 0.1$). The triangle denotes an outlier.

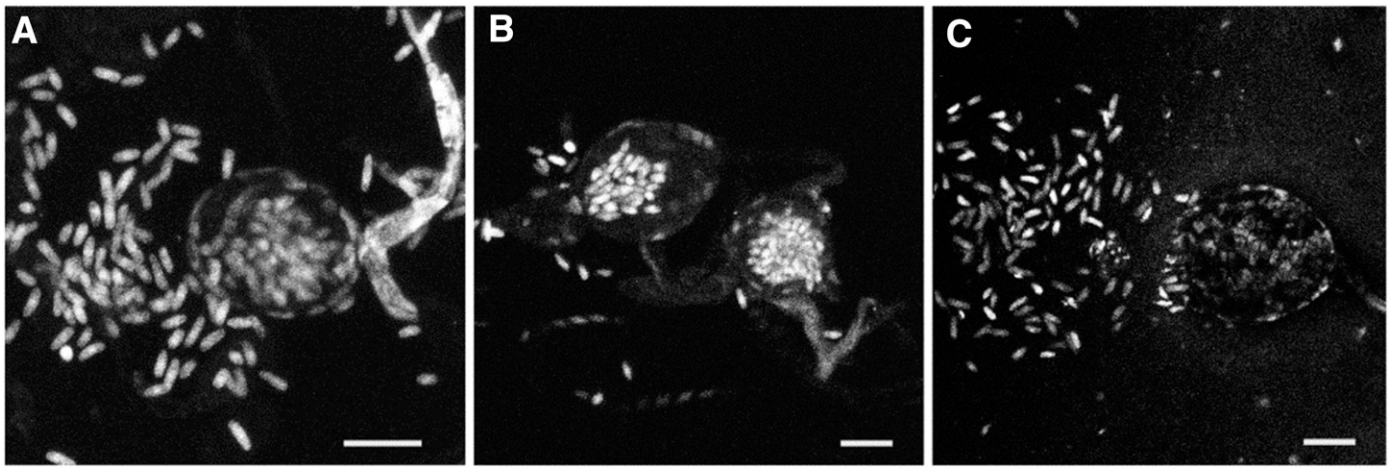

Fig. 4. Pycnidia of transformant J1 of *Ampelomyces* strain RS1-a developed inside conidiophores of *Blumeria graminis* f. sp. *hordei* on barley, 10 days postinoculation. Samples were observed with a confocal laser scanning microscope and images of optical slices were used to prepare the z-stacked projections shown here. **A,** A mature pycnidium produced in the foot cell of a conidiophore and releasing conidia. **B,** Two intracellular pycnidia; release of conidia was observed from the one on the left. **C,** An intracellular pycnidium that has already released most of its conidia. All images show that the green fluroescent protein signal is very weak, or cannot be detected, from the cells of the intracellular pycnidial walls, whereas this signal is always strong from conidia. Bar = 20 µm.

Second, the improved visualization of *Ampelomyces* structures made it possible to perform experiments that focused on deciphering the controversial environmental fate of the *Ampelomyces* fungus (Brimner and Boland 2003; Kiss 2004) before and after its well-known mycoparasitic relationship with powdery mildew colonies. Inoculations of powdery mildew-free cucumber leaves with *Ampelomyces* strains revealed that these mycoparasites can persist up to 21 days on host plant surfaces without being in contact with their mycohosts. After 21 days, the *Ampelomyces* strain was still able to initiate mycoparasitism (i.e., penetrate powdery mildew structures that were provided experimentally on these plants). This has not been previously shown, and the general assumption was that actively growing powdery mildew colonies are always needed for the establishment of *Ampelomyces* mycoparasites (Falk et al. 1995b; Kiss 2008; Kiss et al. 2004; Sztejnberg et al. 1989). This result is also important from a biocontrol perspective because it indicates that commercial *Ampelomyces* biofungicide products sprayed on those parts of the treated crops that are not infected with powdery mildew at the time of the application may not be completely wasted. Those amounts of biofungicide may still have an impact on controlling powdery mildew infections, in a preventive way, because they can initiate mycoparasitism of powdery mildew colonies that may appear later on the respective plant surfaces. However, our results have also indicated that a part of the *Ampelomyces* inoculum was lost during the up to 21-day-long powdery mildew-free period on host plant surfaces. After the establishment of powdery mildew colonies on these plants, the level of mycoparasitism was much lower and patchy compared with plants that were treated with *Ampelomyces* strains when large leaf areas were already covered with powdery mildew, and the mycoparasites could readily attack powdery mildew colonies. This was not surprising because the 10-day-old powdery mildew

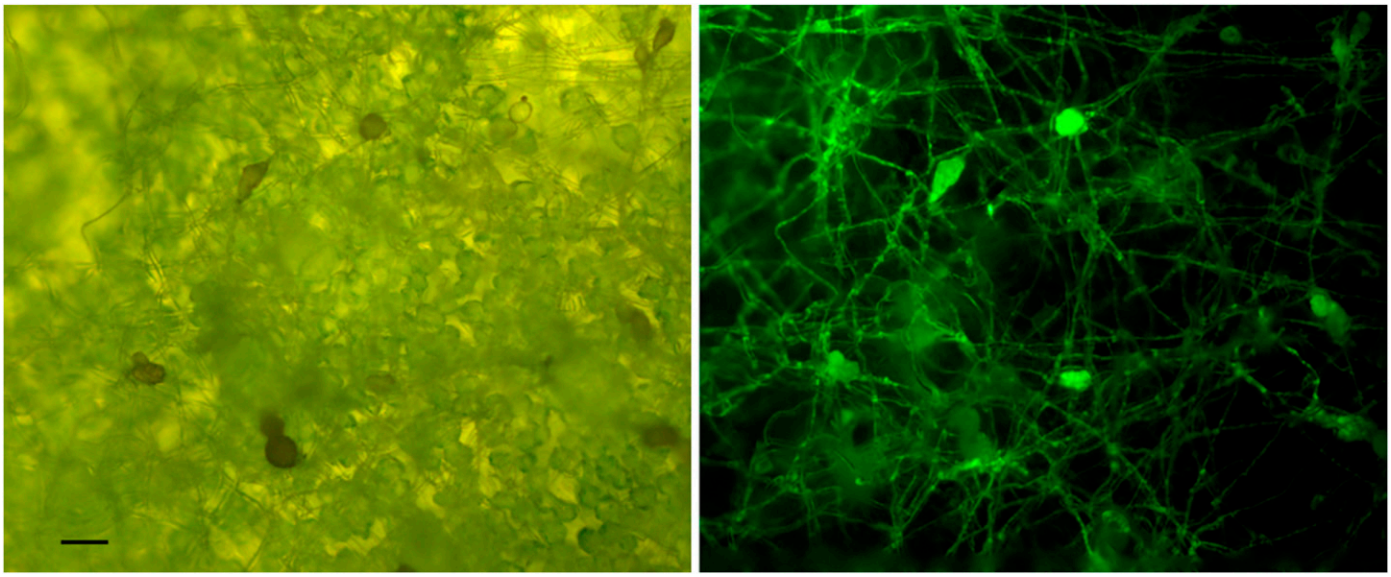

Fig. 6. Intracellular hyphae and pycnidia of transformant B3 of *Ampelomyces* strain RS1-a developed in young, 10-day-old colonies of *Podosphaera xanthii* on cucumber. Conidia of the transformant were sprayed on cucumber plants 4 days before the powdery mildew inoculum was dusted onto these. Left: bright field; right: fluorescence microscopy. Bar = 50 µm.

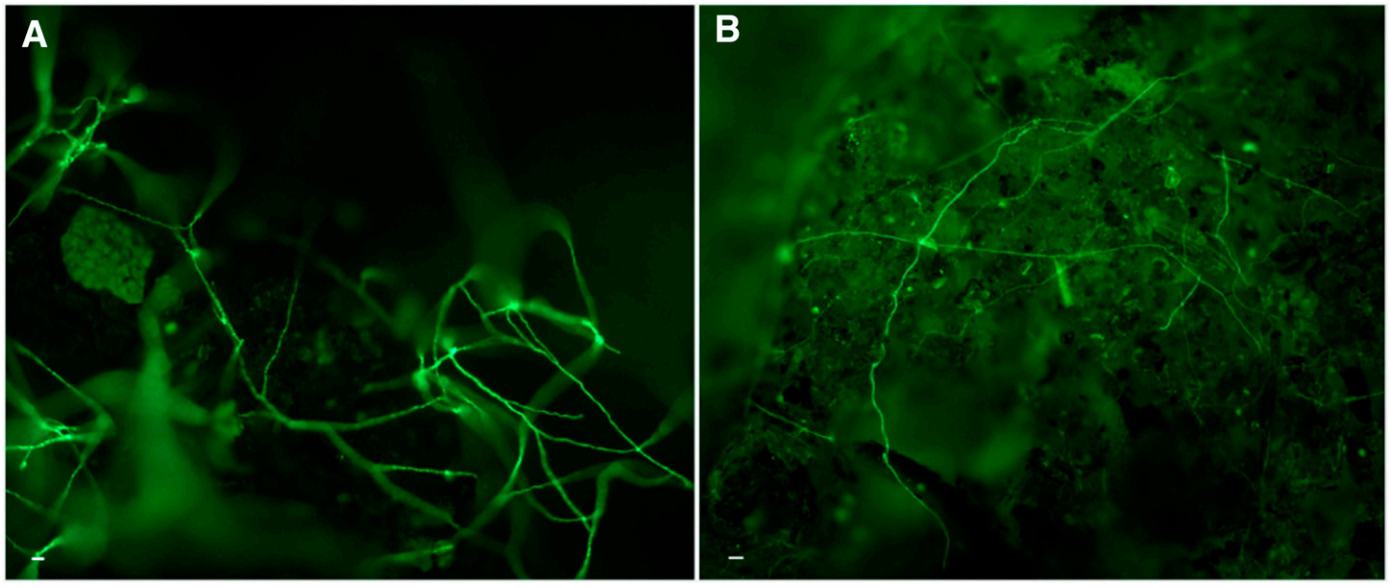

Fig. 7. Hyphae of transformant B3 of *Ampelomyces* strain RS1-a in plates with autoclaved soil, 30 days postinoculation. **A,** Aerial hyphae of the transformant. **B,** Hyphae attached to a soil particle. Bar = 20 µm.

colonies developed on those experimental plants were too young to support more mycoparasitism.

Two experiments were carried out to decipher what happens to *Ampelomyces* fungi on fallen senescent and decomposing powdery mildew-infected leaves on the ground, and in the soil, during the postmycoparasitic phase of their life cycle. When powdery mildew-infected leaf pieces bearing transformants' pycnidia were placed on the soil in pots, in a nonsterile environment, fluorescent microscopy revealed that the GFP signal in samples started to decrease after 10 days and disappeared after 30 days. This was interpreted as a reduction and the end of the GFP gene expression (i.e., transformants' active metabolism), respectively. Most importantly, saprobic production of new pycnidia, and thus new sporulation, was not observed in the decomposing leaf tissues and adjacent soil samples or in the second experiment that consisted of inoculations of autoclaved soil volumes with *Ampelomyces* mycoparasites. The autoclaved soil remained wet until the end of the 30-day experiment; this was regarded as a favorable condition for saprobic pycnidial production. *Ampelomyces* pycnidia were also not detected on powdery mildew-free leaves during our experiment targeting the persistence of these mycoparasites on host plant surfaces in the absence of their mycohosts. In fact, in this study, saprobic development of *Ampelomyces* pycnidia was observed only in culture, similar to earlier works (Falk et al. 1995a, b; Legler et al. 2016; Liang et al. 2007; Liyanage et al. 2018; Park et al. 2010; Pintye et al. 2012, 2015; Sztejnberg et al. 1989). According to this study, saprobic *Ampelomyces* development prior to the mycoparasitic phase of its life cycle was limited to hyphal growth on powdery mildew-free plant surfaces, as well as to local metabolic activity, without any signs of extensive new hyphal growth and new pycnidial production, in or around decomposing powdery mildew-infected leaf tissues during the postmycoparasitic phase. These results indicated that *Ampelomyces* strains, similar to powdery mildews, occupy a niche in the phyllosphere and act there primarily as intracellular mycoparasites of powdery mildews. Moreover, our results showed that the *Ampelomyces* strains were not able to sporulate in the absence of their mycohosts, in either the phyllosphere or the soil level. They may persist in the environment without their mycohosts; based on these results, it is unlikely that they are able to colonize niches other than powdery mildew colonies. Therefore, as suggested earlier (Kiss 2004), concerns regarding the presumed impact of *Ampelomyces* mycoparasites on soil microbiota (Brimner and Boland 2003) remain unconfirmed. Overwintering of *Ampelomyces* fungi as saprobically produced pycnidia in the leaf debris, hypothesized in the early mycological literature (De Bary 1870; Yarwood 1939), has not been confirmed either. All experimental studies on *Ampelomyces* overwintering forms have revealed that these were linked to powdery mildew structures (Falk et al. 1995a; Kiss et al. 2011; Marboutie et al. 1995; Szentiványi and Kiss 2003).

To develop *Ampelomyces* strains constitutively expressing GFP, we utilized ATMT, "a silver bullet in a golden age of functional genomics" (Idnurm et al. 2017). The transformation ratios were similar to those achieved in other studies (Li et al. 2005). GFP fluorescence and hygromycin B resistance of transformants indicated that the heterologous genes and regulator regions provided on the pCBCT plasmid were functional in both *Ampelomyces* strains. These transformants have maintained their hygromycin B resistance and GFP fluorescence for almost 3 years of subculturing in absence of selection pressure, being mitotically stable. Pycnidia of transformants developed in culture and also in powdery mildew conidiophores produced numerous conidia; these were also fluorescent. This has also indicated the mitotic stability of the integrated transgenes. Cells of the *Ampelomyces* pycnidial walls were the only structures in transformants that did not exhibit a strong GFP signal, as revealed by CLSM.

All of the mycoparasitic tests showed that transformants penetrated powdery mildew colonies and sporulated inside powdery mildew conidiophores similar to the wild-type strain. Quantitative data revealed that the level of mycoparasitism did not differ in transformants and the wild-type strain. This indicated that none of the genes that play a role in mycoparasitism were disrupted during GFP transformation. Significant differences ($P \leq 0.004$) were, however, detected in the growth rate of some transformants in culture compared with the wild-type strain. Therefore, transformation may have still had an identifiable effect on the target organisms. Most transformants had a single copy of the T-DNA in their genome; the significant differences in colony growth rate could not be linked to the number of T-DNA copies in different transformants. Further studies can utilize those transformants that had a single T-DNA copy and did not exhibit any significant differences in their cultural characteristics compared with the wild-type strain.

The transformation method was equally successful in two phylogenetically distinct *Ampelomyces* strains that were markedly different from a physiological point of view, as well: RS1-a has always intensively sporulated in culture and has already been selected as a potential BCA (Legler et al. 2016), whereas GYER was characterized by poor and only occasional sporulation in culture during this project, although it did sporulate well prior to this study (Legler et al. 2016). This may indicate that ATMT could be used to transform phylogenetically and physiologically diverse *Ampelomyces* strains. Such transformations may target functional genomics analyses of genes involved in mycoparasitism. A transcriptome analysis of an *Ampelomyces* strain (Siozios et al. 2015) has already provided a useful database for such studies. Therefore, the present work, in addition to shedding light on largely unknown aspects of the life cycle of a widespread mycoparasite, has also established the framework for a molecular genetic toolbox to be used in future studies on the genus *Ampelomyces*.

ACKNOWLEDGMENTS

We thank Csilla Rohm, Ildikó Csorba, and Endre Tóth for technical assistance and Klára Mészáros for providing the barley genotype used in this work.

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