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A coumarin exudation pathway mitigates arbuscular mycorrhizal incompatibility in *Arabidopsis thaliana*

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Abstract

Key message **Overexpression of genes involved in coumarin production and secretion can mitigate mycorrhizal incompatibility in nonhost Arabidopsis plants. The coumarin scopoletin, in particular, stimulates pre-penetration development and metabolism in mycorrhizal fungi.**

Abstract Although most plants can benefit from mutualistic associations with arbuscular mycorrhizal (AM) fungi, nonhost plant species such as the model *Arabidopsis thaliana* have acquired incompatibility. The transcriptional response of Arabidopsis to colonization by host-supported AM fungi switches from initial AM recognition to defense activation and plant growth antagonism. However, detailed functional information on incompatibility in nonhost–AM fungus interactions is largely missing. We studied interactions between host-sustained AM fungal networks of *Rhizophagus irregularis* and 18 Arabidopsis genotypes affected in nonhost penetration resistance, coumarin production and secretion, and defense (salicylic acid, jasmonic acid, and ethylene) and growth hormones (auxin, brassinosteroid, cytokinin, and gibberellin). We demonstrated that root-secreted coumarins can mitigate incompatibility by stimulating fungal metabolism and promoting initial steps of AM colonization. Moreover, we provide evidence that major molecular defenses in Arabidopsis do not operate as primary mechanisms of AM incompatibility nor of growth antagonism. Our study reveals that, although incompatible, nonhost plants can harbor hidden tools that promote initial steps of AM colonization. Moreover, it uncovered the coumarin scopoletin as a novel signal in the pre-penetration dialogue, with possible implications for the chemical communication in plant–mycorrhizal fungi associations.

Keywords Arbuscular mycorrhizae · Symbiotic incompatibility · Plant defense · Coumarin secretion · Molecular dialogue · Plant hormones

Introduction

In nature, most land plants are compatible hosts for arbuscular mycorrhizal (AM) fungi (Brundrett and Tedersoo 2018). These plants employ a sophisticated molecular dialogue with their AM fungal partners to establish beneficial symbioses (Bucher et al. 2014). The AM fungi are obligate biotrophs that are widespread throughout the globe (Davison et al. 2015). They constitute keystone taxa of belowground microbiomes (Banerjee et al. 2018) and contribute to improve plant yield and stress tolerance, nutrient and carbon cycling, soil structure, ecosystem multifunctionality, and agriculture sustainability (Jung et al. 2012; Siddiky et al. 2012; Van der Heijden et al. 2015; Bitterlich et al. 2018; Rillig et al. 2019). However, during plant evolution, approximately 29% of vascular species developed symbiotic incompatibility with AM fungi (Brundrett and Tedersoo 2018). Among these nonhost

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species is the model plant *Arabidopsis thaliana* (hereafter Arabidopsis), but also various economically important vegetables (e.g. spinach, turnip, cabbage, and broccoli) (Wang and Qiu 2006; Brundrett and Tedersoo 2018; Cosme et al. 2018). Global cash crops such as sugar beet (Hajiboland et al. 2020) and rapeseed (Poveda et al. 2019), one of the largest global sources of vegetable oil, are also considered nonhosts for AM fungi. Understanding the mechanisms that control symbiotic incompatibility is paramount to evaluate the biological factors that delimit AM symbiosis and could help to improve symbioses in sustainable agriculture. Yet, despite the comprehensive molecular toolkit of Arabidopsis, we know little about nonhost regulators of mycorrhizal incompatibility.

Although the members of the Brassicales are generally considered as nonhost plants (Brundrett and Tedersoo 2018), some level of AM colonization has been documented for a number of species in this order (Tommerup 1984; Peterson et al. 1985; Orłowska et al. 2002; Cosme et al. 2014; Bueno et al. 2018). In Arabidopsis seedlings, the rhizosphere microbiome was found to be enriched for endemic AM fungi in a coumarin-dependent manner relative to the surrounding bulk soil. This suggests that in the pre-penetration stage, AM fungi can be attracted to Arabidopsis roots and that root-secreted coumarins may play a role in this process (Stringlis et al. 2018b; Fernández et al. 2019). Experimental attempts to associate Arabidopsis with AM fungal propagules led only to limited colonization (between 0 and 15% of the root system) at late stages of plant development (Kruckelmann 1975; DeMars and Boerner 1996; Veiga et al. 2013; Poveda et al. 2019). However, these levels increased up to 43% when Arabidopsis roots were exposed to a pre-established AM mycelium nurtured by compatible host plants (Veiga et al. 2013). Similar to compatible hosts, colonization of Arabidopsis is characterized by activation of strigolactone (SL) biosynthesis genes in the roots upon detection of AM fungi (Fernández et al. 2019). Additionally, formation of hyphopodia-like structures for fungal attachment to the root surface, fungal penetration of roots, and intraradical hyphal proliferation can be observed (Veiga et al. 2013; Cosme et al. 2018; Fernández et al. 2019). The interactions between nonhost Arabidopsis plants and AM fungi (as well as ectomycorrhizal fungi) can lead to non-nutrient-based benefits for the plant, such as the onset of induced system resistance against foliar pathogen or insect attack (Fernández et al. 2019; Vishwanathan et al. 2020). However, subsequent steps in AM formation are impaired and fungal morphological markers of symbiotic nutrient exchange, i.e. intracellular arbuscules, hyphal coils or arbusculated coils, are either very rare or absent inside Arabidopsis roots (Cosme et al. 2018). Consistently, fungal nutrient transporter genes that are generally induced during AM symbiosis were not expressed inside colonized Arabidopsis roots (Fernández et al. 2019). Moreover, various genes that are part of the symbiotic toolkit in legume host plants are absent from the genome of Arabidopsis, which might largely explain its degree of symbiotic incompatibility (Delaux et al. 2014). Yet, the molecular signals used by plants and AM fungi to directly detect and evaluate their degree of incompatibility are still largely unknown (Cosme et al. 2018).

To obtain insight into the mechanisms of incompatibility, we analyzed direct interactions of AM fungi with Arabidopsis mutant and transgenic lines altered in specific genes involved in nonhost penetration resistance, coumarin secretion, and defense or growth hormone signaling. Among a diverse array of small molecules secreted by Arabidopsis roots, coumarins display an outstanding ability to shape the assembly of the whole rhizosphere microbiome (Stringlis et al. 2018b; Voges et al. 2019). The coumarin scopoletin, for instance, possesses a selective antimicrobial activity (Stringlis et al. 2019) and can operate as a first line of root defense by selectively suppressing pathogenic fungi, while not affecting other rhizosphere microbial community members (Stringlis et al. 2018b; Stassen et al. 2021). Hence, root-secreted coumarins might play a similar suppressive role on AM fungi in Arabidopsis. However, they could alternatively play a positive role, as the enrichment for endemic AM fungi on Arabidopsis roots was dependent on a functional coumarin exudation pathway (Stringlis et al. 2018b; Fernández et al. 2019). Coumarin biosynthesis and secretion is induced upon colonization of roots by specific beneficial rhizobacteria, but also in response to depletion of mineral nutrients (Stringlis et al. 2018b), such as iron (Fe) and phosphate (Pi) in the soil environment. Upon secretion, coumarins function in the mobilization and uptake of these nutrients, therewith alleviating the nutrient stress (Sisó-Terraza et al. 2016; Ziegler et al. 2016; Tsai and Schmidt 2017a; Chutia et al. 2019). The transcription factor MYB72 is an important regulator of coumarin biosynthesis (Zamioudis et al. 2014; Stringlis et al. 2018b). It co-regulates a number of genes in Arabidopsis roots associated with coumarin production and secretion, including *F6'H1,* encoding an important coenzyme in the biosynthesis of coumarins (Kai et al. 2008; Schmid et al. 2014), and *BGLU42,* encoding a β-glucosidase (Zamioudis et al. 2014). In Arabidopsis, MYB72-dependent BGLU42 deglycosylation activity was shown to be important for the conversion of the major coumarin scopolin into scopoletin and its subsequent secretion into the rhizosphere (Zamioudis et al. 2014; Stringlis et al. 2018b). *MYB72* overexpression upregulates coumarin biosynthesis gene *F6'H1* but does not constitutively induce the expression of *BGLU42* (Zamioudis et al. 2014), suggesting that MYB72 acts together with one or more other transcription factors in the regulation of *BGLU42* (Zamioudis et al. 2014).

The detection of microbes by Arabidopsis roots follows an immune recognition of conserved microbe-associated

molecular patterns (MAMPs) (Yu et al. 2019b). Upon recognition, the hormone salicylic acid (SA) generally acts as a master regulator of defense against invasion by biotrophic pathogens, while the hormones jasmonic acid (JA) and ethylene (ET) regulate the production of defensive glucosinolates and defense against necrotrophic pathogens (Pieterse et al. 2012; Pangesti et al. 2016). MAMPs of pathogens, however, are not fundamentally different from those of beneficial microbes (Stringlis et al. 2018a; Yu et al. 2019b). As for the adapted pathogens, beneficial microbes, such as plant growth-promoting rhizobacteria and AM fungi evolved means to evade or suppress the immune system of compatible hosts (Toro & Brachmann, 2016; Yu et al. 2019a). However, the invasion by AM fungi in Arabidopsis activates rather than suppresses root immunity, leading to an induction of SA-, JA- and glucosinolate-related defenses (Fernández et al. 2019; Poveda et al. 2019). This suggests that immune suppression mechanisms of AM fungi do not function in nonhost Arabidopsis roots, resembling the failure of non-adapted pathogens to suppress plant immunity in which e.g. the penetration (PEN) genes act as central components of nonhost cell wall-based defense (Johansson et al. 2014). Although failure to suppress plant immunity might potentially contribute to limiting AM fungal colonization inside Arabidopsis roots, functional information on the mechanisms underlying nonhost defense against AM fungi is virtually lacking. The induction of nonhost defenses by AM mycelium associates with growth suppression of Arabidopsis (Veiga et al. 2013; Fernández et al. 2019). Whether AM-mediated activation of defenses limits fungal colonization and/or induces growth-defense tradeoffs in nonhost plants (Huot et al. 2014; Van Butselaar and Van den Ackerveken 2020) remains to be tested.

To gain detailed insight in mycorrhizal incompatibility, we investigated the molecular basis of incompatibility and antagonism between the AM fungus *Rhizophagus irregularis* and the nonhost plant Arabidopsis. In particular, we addressed the following main questions: (1) does nonhost penetration resistance and coumarin secretion influence colonization of nonhost Arabidopsis roots by host-nursed AM fungi? and (2) does defense and growth hormone signaling regulate host-nursed AM colonization and growth-defense tradeoffs in nonhost Arabidopsis plants? From our study of the effect of AM fungi on a selection of 18 Arabidopsis genotypes, the plant-derived coumarin scopoletin emerged as a positive modulator of nonhost roots–AM fungi interactions.

Materials and methods

Arbuscular mycorrhizal fungus

Rhizophagus irregularis (Błaszk., Wubet, Renker & Buscot) was used as a model AM fungus that is incompatible with the nonhost Arabidopsis accession Col-0 (Veiga et al. 2013; Fernández et al. 2019). For experiments conducted under in vivo conditions, we used inoculum of *R. irregularis* strain BEG21 produced as previously described (Veiga et al. 2013). For experiments conducted under in vitro conditions, we used spores of *R. irregularis* strain MUCL41833 obtained from the Glomeromycota in vitro collection (GINCO, Belgium).

Plants

Arabidopsis thaliana (L.) Heynh. accession Col-0 was used as the wild-type Arabidopsis genotype incompatible for *R. irregularis* (Veiga et al. 2013; Fernández et al. 2019). The mutant and transgenic lines of Arabidopsis used in this study were all in the Col-0 background (Table S1). For Arabidopsis genotypes impaired in coumarin production and secretion (Stringlis et al. 2018b), we used mutants *f6'h1-1,* which is blocked in scopoletin biosynthesis (Kai et al. 2008), *myb72-2* (Van der Ent et al. 2008), which is impaired in the production of the coumarins scopoletin and esculetin (Stringlis et al. 2018b), and *bglu42-1* (Zamioudis et al. 2014), which is impaired in the deglycosylation of scopolin and consequently in the secretion of its aglycon scopoletin (Stringlis et al. 2018b). Additionally, the overexpression lines *35S:YFP-MYB72* (hereafter *35S:MYB72*) and *35S:YFP-BGLU42* (hereafter *35S:BGLU42*) (Zamioudis et al. 2014) were used. *35S:MYB72* displays constitutive upregulation of virtually all genes of the phenylpropanoid pathway leading to coumarin biosynthesis (Zamioudis et al. 2014). *35S:BGLU42* plants were used for their enhanced potential to deglycosylate scopolin and secrete scopoletin (Zamioudis et al. 2014). For Arabidopsis genotypes affected in hormone-regulated defenses, we used SA biosynthesis mutants *sid2-1* (Nawrath and Métraux 1999) and *eds5-1* (Nawrath et al. 2002), SA signaling mutant *npr1-1* (Cao et al. 1994), JA biosynthesis mutant *dde2-2* (Von Malek et al. 2002), and ET signaling mutant *ein2-1* (Guzmán and Ecker 1990). For Arabidopsis genotypes compromised in nonhost defense against non-adapted microbial invaders, we used the penetration double mutant *pen1-1pen2-1* (Johansson et al. 2014), the enhanced disease susceptibility mutant *eds1-2* (Falk et al. 1999; Johansson et al. 2014), and the hypersensitive reaction mutant *edr1-1* (Frye and Innes 1998; Hiruma et al. 2011); the latter also displays enhanced disease resistance to biotrophs and enhanced susceptibility to necrotrophs. For Arabidopsis genotypes affected in growth hormone pathways, we used the auxin perception triple mutant *tir1afb2afb3* (Dharmasiri et al. 2005), the brassinosteroid (BR) biosynthesis mutant *rot3-1* (Kim et al. 2005), and the gibberellic acid (GA) biosynthesis double mutant *ga20ox1-1ga20ox2-1* (Rieu et al. 2008). Additionally, we used the transgenic lines *35S:CKX3-9* and *P10:CKX3-10*, which

produce a reduced global or root-specific level of cytokinin (CK), respectively (Werner et al. 2010). As compatible hosts for *R. irregularis*, we used *Medicago truncatula* Gaertn A17 and Ri T-DNA-transformed roots of *Daucus carota* L. for in vivo and in vitro bi-compartmented experiments, respectively.

In vivo bi-compartmented microcosm experiments

To study Arabidopsis susceptibility to colonization and antagonism by host-supported *R. irregularis*, we established in vivo bi-compartmented microcosm systems as previously described (Fernández et al. 2019), with minor modifications. Briefly, each system consisted of a microcosm with two equal compartments (0.5 L), separated with a 30-µm nylon mesh that physically separated the roots of *M. truncatula* from those of Arabidopsis, but allowed growth of AM mycelium between compartments. In each microcosm, two *M. truncatula* seedlings were grown in the host compartment, next to six seedlings of a specific Arabidopsis genotype in the nonhost compartment. For all tested Arabidopsis genotypes, each of the biological replicates consisted of a pooled sample of six plants of Arabidopsis and two neighboring plants of *M. truncatula* co-cultivated in the same microcosm. A total of six independent bi-compartmented microcosm experiments were performed to study the effects of nonhost resistance, coumarins, and defense and growth hormones on mycorrhizal incompatibility (Table S1). Exp. 1 addressed the effects of nonhost resistance and coumarins. Exp. 2, 3 and 4 addressed the effects of coumarins. Exp. 5 and 6 addressed the effects of defense and growth hormones, respectively. Exp. 1, 2, 5 and 6 had each 48 microcosms, corresponding to four replicates of six different Arabidopsis genotypes inoculated or not with *R. irregularis*. Exp. 3 and 4 had each 24 microcosms, corresponding to eight replicates of three different Arabidopsis genotypes inoculated with *R. irregularis*. Growth parameters such as substrate mixture, inoculation treatment with or without *R. irregularis*, seed surface sterilization and germination, plant transplantation, watering and nutrient solution regimes, growth chamber conditions (photoperiod, light intensity, temperature and relative humidity), and plant age at final harvest were similar to those used by Fernández et al. (2019); except for Exp. 4, where the photoperiod was 16 h instead of 10 h. At harvest, the average shoot fresh weight per plant in each compartment was determined to calculate the plant growth response to *R. irregularis* as described below. The root systems of all plants in each compartment were collected and cleaned from substrate particles for subsequent microscopic analysis of AM fungal colonization as described below. The Col-0 and *M. truncatula* plants were considered as the nonhost and host controls for AM colonization, respectively.

In vivo pot experiments

We established two in vivo pot experiments using direct inoculation with *R. irregularis*, either with a short-day photoperiod of 10 h (Exp. 7) or a long-day photoperiod of 16 h (Exp. 8). Plants of Col-0 and *M. truncatula* were included as nonhost and host controls for AM colonization, respectively. Arabidopsis and *M. truncatula* seeds were surface sterilized as previously described (Fernández et al. 2019) and sown directly onto 60-mL pots containing a sterilized soil mixture supplemented with 10% v:v of *R. irregularis* inoculum or a mock control (as in the bi-compartmented microcosm experiments). After 3 d of seed stratification at 4 °C in the dark, the pots were transferred to a growth room according to photoperiod treatment. Light intensity, temperature and relative humidity were similar to the in vivo bi-compartmented microcosm experiments. Two weeks after germination, plants were thinned to one plant per pot. Each plant genotype, i.e. Col-0, *35S:MYB72*, *35S:BGLU42* or *M. truncatula*, had five replicates in each experiment. The pots were watered twice a week until saturation. In order to limit the availability of Pi, plants were fertilized once a week with 1 mL of a modified half-strength Hoagland solution containing 25% of the standard KH_2PO_4 concentration. Plants were harvested after 11 weeks of growth. Their roots were collected and cleaned from substrate particles for subsequent microscopic analysis of AM fungal colonization as described below.

AM fungal colonization

Root samples of all Arabidopsis genotypes and of *M. truncatula* grown in vivo were analyzed at the end of each experiment to determine the presence of AM fungal structures. To this end, root fragments of each biological replicate were stained with trypan blue solution (Phillips and Hayman 1970). Colonization by *R. irregularis* was analyzed at ×200 magnification on 20 one-cm-long root fragments per biological replicate using the magnified intersections method (McGonigle et al. 1990). The presence of hyphae on the root surface (hereafter epiphytic hyphae) or hyphae inside the roots (hereafter endophytic hyphae) as well as arbuscules or vesicles was used to determine the percentage of root length colonization.

In vitro bi-compartmented plate experiments

Two different in vitro bi-compartmented plate systems were used to expose the AM fungus to Arabidopsis roots or scopoletin application, respectively. To expose the AM fungus in vitro to Arabidopsis roots, two experiments were conducted using a large bi-compartmented plate system (Koffi and Declerck 2015). Briefly, this system consisted of the

lid of a Petri dish with a diameter of 55 mm placed inside a larger Petri dish with a diameter of 145 mm, creating a host compartment inside a nonhost compartment. Each host compartment was filled with modified Strullu–Romand medium (MSR; Declerck et al. 1998). The nonhost compartment was filled with MSR medium depleted of sucrose and vitamins (Voets et al. 2005). A root fragment of transformed *D. carota* roots was transplanted into each host compartment and inoculated with *R. irregularis*. The two independent experiments were Exp. 9 in which the AM fungus was exposed to Arabidopsis roots for 6 weeks, and Exp. 10 in which the AM fungus was exposed to Arabidopsis roots for 9 weeks. Plates were initially incubated inverted at 27 °C in the dark for three months to fully develop the *D. carota*-supported mycorrhizal network in the nonhost compartment. Subsequently, six Arabidopsis Col-0, *35S:MYB72,* or *35S:BGLU42* seedlings were transplanted into each nonhost compartment. Seedlings of Col-0, *35S:MYB72* and *35S:BGLU42* were germinated and pre-grown in vitro as described (Fernández et al. 2019). For each Arabidopsis genotype, four independent in vitro bi-compartmented plate systems were used in each experiment. To allow free development of Arabidopsis aerial parts after transplantation, each bi-compartmented plate system was covered with an adapted round, gamma radiation-sterilized microbox container. The bi-compartmented plate systems were then placed in a growth chamber (21 °C; 16 h of photoperiod; 300 µmol m^{-2} s^{-1} of light intensity; 70% of relative humidity) until harvest for subsequent determination of fungal metabolic activity as described below. Prior to harvest of Exp. 10, the length density of the extraradical hyphae in the nonhost compartment was determined using the grid intersection method (Newman 1966).

To expose the AM fungus to different concentrations of scopoletin, we used *D. carota* root organ cultures in an in vitro bi-compartmented plate system (Exp. 11). This system consisted of a 90-mm diameter Petri dish containing a host compartment with Ri T-DNA-transformed *D. carota* roots inoculated with *R. irregularis,* and a hyphal compartment as previously described (Goh et al. 2019), with a minor modification. When the AM fungus was fully established in the hyphal compartment, the MSR medium in the hyphal compartment was partially removed, leaving a one-cm-wide strip of medium with pre-established mycelium that remained connected to the host compartment. The area with removed medium was then re-filled with MSR medium depleted of sucrose and vitamins and supplemented with scopoletin or a mock control. To add scopoletin to the MSR medium, scopoletin powder (Sigma-Aldrich) was dissolved in purged 70% ethanol and mixed with the MSR medium at 45 °C to reach a final scopoletin concentration of 0.5 or 7.7 µg mL^{-1}. The mock control medium was supplemented with similar amounts of purged 70% ethanol solution. To allow re-growth of the AM fungus on the scopoletin-containing MSR medium, the plate systems were incubated in the dark at 27 °C in an inverted position. Eight weeks later, the hyphal length density of the re-grown mycelium was determined using the grid intersection method (Newman 1966). The re-grown mycelium was then harvested for subsequent determination of fungal metabolic activity as described below.

AM fungal metabolic activity

At the end of each in vitro bi-compartmented plate experiment, the hyphal compartments were sampled. For the large in vitro bi-compartmented plate system used to expose mycelium to Arabidopsis roots (described above), the roots were first carefully removed, after which the whole hyphal compartment was sampled. For the in vitro bi-compartmented plate system use to exposed re-growing mycelium to scopoletin (described above), the re-grown area of the hyphal compartment was sampled. The sampled medium was liquefied using sodium citrate buffer (10 mM, pH 6.0) (Doner and Bécard 1991) and filtered through a 40-µm nylon mesh to harvest the mycelium. The mycelium was then stained with nitro blue tetrazolium chloride and counterstained with acid fuchsin to determine the presence of succinate dehydrogenase (SDH), as marker for metabolic activity (Saito et al. 1993). The stained mycelium was mounted on slides for microscopic observation. The hyphae were classified into two categories: SDH-active and -inactive, corresponding to blue-black- and pink-stained hyphae, respectively. The percentage of hyphal length with SDH activity was quantified by cross intersect using an adapted magnified intersect method (McGonigle et al. 1990) with ×400 magnification and 200 intersections per replicate.

In vitro spore germination bioassays

Spores of *R. irregularis* were placed singly at the center of a Petri dish (3-cm diameter) containing 3 mL of solidified MSR medium depleted of sucrose and vitamins. The plated spores were incubated overnight at 25 °C in the dark to allow germ tube initiation. Spores with 1-day-old germ tubes were randomly distributed among scopoletin concentration treatments and the mock control. The mock control consisted of a purged 70% ethanol solution. To prepare the scopoletin concentration treatments, scopoletin powder (Sigma-Aldrich) was dissolved in purged 70% ethanol at a concentration of 2000 µg mL^{-1}. This solution was then diluted in purged 70% ethanol using a step-wise 1:100 dilution factor until 0.00002 µg mL^{-1}. To apply the scopoletin treatments, a Whatman® paper disk (6 mm in diameter, Sigma-Aldrich) was placed at the edge of the plate at a distance of approximately 6 mm from the germinating spore. The paper disk was then loaded with 15 µL of a scopoletin

dilution or a mock control solution. After scopoletin application, the spores were incubated at 25 °C in the dark. In order to measure hyphal length and number of newly formed hyphal tips, we collected pictures of the germinating spores using a compact microscope. In Exp. 12, pictures were collected at 1, 3 and 5 days after scopoletin application, while in Exp. 13 pictures were collected at 7 days. The length of the hyphae was determined using ImageJ software.

Aboveground mycorrhizal growth response

At the end of Exp. 1, 2, 5 and 6 described above, the shoot parts of the six Arabidopsis plants in each biological replicate were cut at the base. The plant shoots were immediately weighted to calculate the average fresh shoot biomass per plant in each biological replicate. To determine the aboveground mycorrhizal growth response (MGR) of the different Arabidopsis lines, we used the following formulas (Veiga et al. 2011):

$$if\ NAM < AM,\ theMGR(\%) = \left(1 - \left(\frac{NAM}{AM}\right)\right) \cdot 100$$

$$if\ NAM > AM,\ theMGR(\%) = \left(-1 + \left(\frac{AM}{NAM}\right)\right) \cdot 100$$

In these formulas, NAM stands for the mean value in each experiment of the aboveground fresh biomass per plant of an Arabidopsis line grown without *R. irregularis*, while AM stands for the aboveground fresh biomass per plant in each biological replicate of the corresponding Arabidopsis line grown in presence of *R. irregularis*.

Statistical analyses

All data were analyzed using RStudio (https://rstudio.com) with R version 3.6.1. Data for AM colonization and mycorrhizal growth response of each Arabidopsis mutant or overexpression line grown in the in vivo bi-compartmented microcosms were compared separately with that of Col-0 using mixed-effects models as previously described (Veiga et al. 2013). Data for AM colonization for plants grown in pots and for the percentage of SDH-active hyphae or spore germination parameters were subjected to one-way ANOVA followed by Duncan's test.

Results

Evidence for a positive role of root-secreted coumarins in the nonhost *Arabidopsis–R. irregularis* interaction

Previously, it was demonstrated that nonhost Arabidopsis roots can to some extent be colonized by *R. irregularis* when the AM network is supported by a neighboring AM host plant, albeit that the resulting nonhost–AM fungus interaction does not lead to a functional symbiosis and even antagonizes growth of the nonhost (Veiga et al. 2013; Fernández et al. 2019). Recent findings show that endemic *Rhizophagus* sp. are enriched in the rhizosphere of coumarin-secreting Arabidopsis plants (Stringlis et al. 2018b), but after invasion of the nonhost roots, further AM fungal ingress seem to be prevented due to the activation of nonhost immune responses (Fernández et al. 2019; Poveda et al. 2019). This prompted us to investigate whether nonhost defense and coumarin secretion can affect symbiotic incompatibility in the nonhost Arabidopsis. To this end, we used a bi-compartmented microcosm system (Fig. S1) in which *R. irregularis* is supported by *M. truncatula* host plants to compare AM fungal colonization in Arabidopsis wild-type Col-0 plants with that in Arabidopsis mutants compromised in nonhost defense against non-adapted microbial pathogens (*pen1pen2*, *eds1*, and *edr1*) and mutants impaired in coumarin biosynthesis and secretion (*myb72* and *bglu42*) (Exp. 1). As expected, microscopic analysis of AM fungal root colonization confirmed the presence of hyphae of *R. irregularis* inside the root cortex (endophytic hyphae) and on the root surface (epiphytic hyphae) of nonhost Col-0 plants (Fig. 1a). However, in contrast to the heavily colonized roots of *M. truncatula* host plants (> 80% of root length colonized by *R. irregularis* with abundant arbuscule and vesicle formation; Fig. S2a, h), the roots of Col-0 had only a minor percentage of root length colonized by endophytic hyphae (Fig. 1b), without detectable arbuscules or vesicles (not shown), confirming the mycorrhizal incompatibility of Col-0. Moreover, the morphological development of *R. irregularis* in Col-0 roots was characterized by aseptate hyphae proliferating along the root surface with sporadic entry points (Fig. 1a). These entry points appeared to

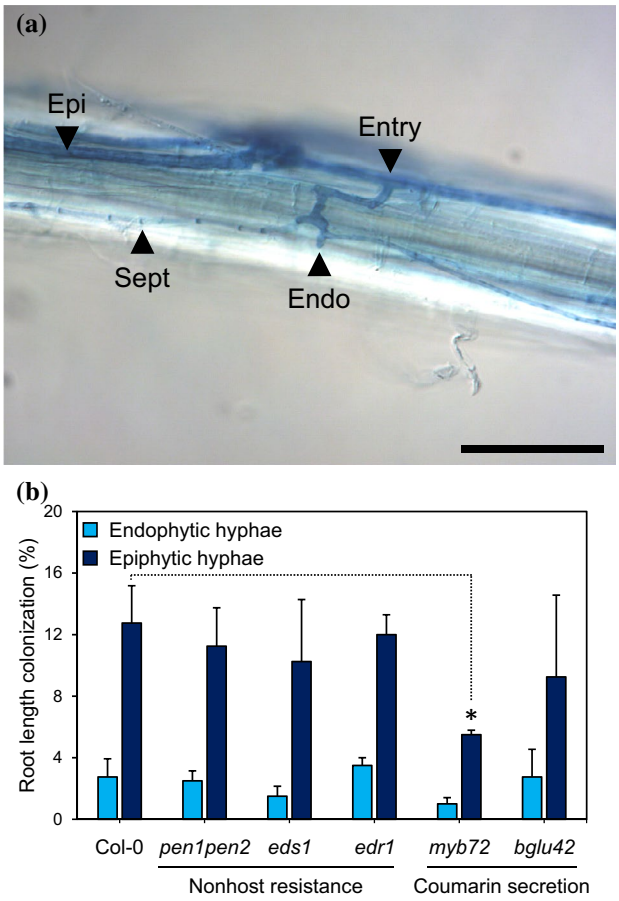

Fig. 1 Colonization by *Rhizophagus irregularis* in nonhost roots of nonhost resistance- and coumarin-related Arabidopsis mutants. **a** Typical colonization phenotype of trypan blue-stained *R. irregularis* in wild-type Arabidopsis roots showing epiphytic aseptate hyphae (Epi) proliferating along the root surface with sporadic entry points (Entry) allowing fungal penetration into the root, followed by intraradical proliferation by relatively thinner endophytic hyphae (Endo), which in some cases became septate (Sept). Bar = 50 µm. **b** Percentage of root length colonized by epiphytic and endophytic hyphae of *R. irregularis* in Arabidopsis mutants compromised in nonhost defense against non-adapted pathogens (*pen1pen2*, *eds1*, *edr1*) or in coumarin biosynthesis and secretion (*myb72* and *bglu42*), compared with that of Col-0. Colonization was analyzed in 11-week-old Arabidopsis plants after 9 weeks of co-cultivation with *R. irregularis* supported by host plants of *Medicago truncatula* in bi-compartmented microcosm systems (Supplementary Fig. S1). Asterisks indicate statistically significant differences compared with Col-0 (mixed-effects models, $P < 0.05$). Mean + SE, $n = 4$

develop mostly between epidermal root cells (Fig. 1a). The endophytic hyphae invading the roots became thinner compared with the epiphytic hyphae, had limited proliferation within the root, and often became septate (Fig. 1a). This morphological development of *R. irregularis* in Col-0 was similar in *pen1pen2*, *eds1*, *edr1*, *myb72*, and *bglu42*. The percentage of root length colonized by endophytic hyphae in *pen1pen2*, *eds1*, *edr1*, *myb72* or *bglu42*, was not significantly different from that of Col-0 (Fig. 1b). Although the percentage of root length colonized by epiphytic hyphae was also not significantly different between Col-0 and *pen1pen2*, *eds1*, *edr1* or *bglu42*, we observed a significant reduction in the proliferation of epiphytic hyphae along the root surface of *myb72* (Fig. 1b). Collectively, these results indicate that none of the tested nonhost resistance genes seem to play a role as a first-line mechanism of mycorrhizal incompatibility. However, the results with *myb72* point to a positive role of MYB72 in the pre-penetration interaction between Arabidopsis and the AM fungus, corroborating earlier findings that endemic *Rhizophagus* sp. are enriched in the rhizosphere of coumarin-producing Arabidopsis plants (Stringlis et al. 2018b). The roots of *M. truncatula* and Arabidopsis plants grown in non-inoculated control microcosms never showed signs of AM fungal colonization (data not shown), demonstrating that the observed AM fungal root colonizations in the inoculated microcosms are caused by *R. irregularis*.

To follow-up on the putative positive effect of coumarins on the Arabidopsis–*R. irregularis* interaction, we conducted a bi-compartmented microcosm experiment (Exp. 2) to compare AM fungal colonization of roots of the coumarin biosynthesis mutants *myb72*, *bglu42* and *f6'h1,* and the overexpression lines *35S:MYB72* and *35S:BGLU42* with that of Col-0. As observed in Exp. 1, the host roots of *M. truncatula* were abundantly colonized by *R. irregularis* (Fig. S2b), whereas the roots of Col-0 had only a minor percentage of root length colonization (Fig. 2a) without detectable arbuscules or vesicles (not shown), again showing that Col-0 is a nonhost for AM colonization but facilitates a low percentage of root colonization when *R. irregularis* is nursed by the host *M. truncatula*. Although in Exp. 1 we observed a significant decline in colonization of the *myb72* roots by epiphytic AM hyphae relative to the already low percentage of root colonization in Col-0 roots (Fig. 1b), this decline was not apparent in Exp. 2 (Fig. 2a). Also, the coumarin exudation and biosynthesis mutants *bglu42* and *f6'h1* did not show a significant difference in AM hyphal colonization compared with that of Col-0 in Exp. 2 (Fig. 2a). Coumarin secretion by wild-type Arabidopsis is induced by limited availability of mineral nutrients such as Fe and Pi (e.g. Chutia et al. 2019). Hence, it is possible that in contrast to Exp. 1, in Exp. 2 the wild-type Col-0 might not have secreted sufficient amounts of coumarins to display any difference with the mutants *myb72*, *bglu42*, or *f6'h1*. What nevertheless can be concluded from the coumarin biosynthesis mutant data is that coumarins are not strictly required for a baseline level of Arabidopsis root colonization by host-nursed AM hyphae. Interestingly, the percentage of root colonization by endophytic hyphae in *35S:BGLU42* was significantly increased compared with that of Col-0 (Fig. 2a), while that of *35S:MYB72* was apparently increased, albeit non-significantly in Exp. 2 ($P = 0.255$; Fig. 2a). Likewise, the percentage of root colonization by

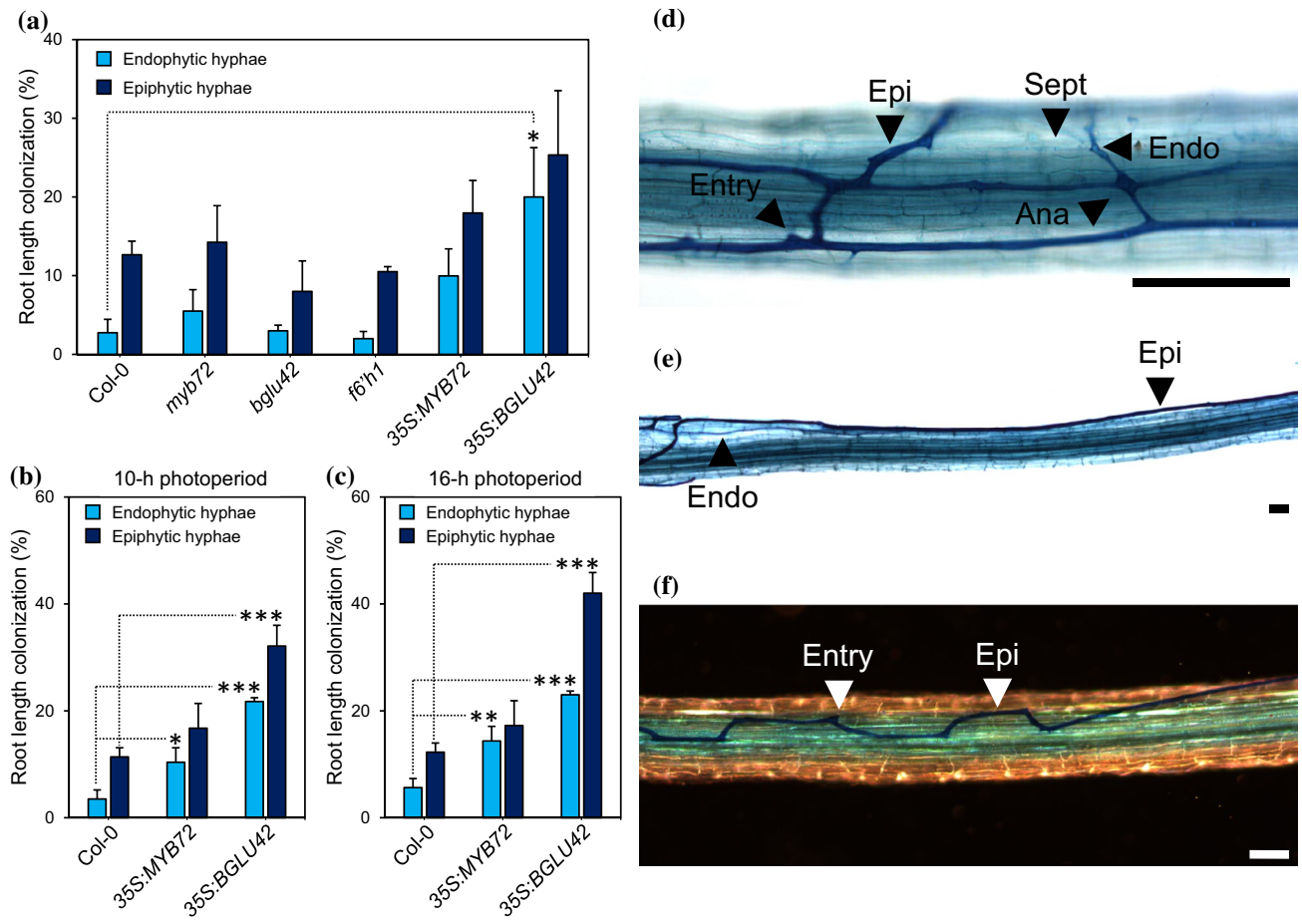

Fig. 2 Colonization by *Rhizophagus irregularis* in nonhost roots of Arabidopsis lines altered in coumarin production and secretion. Colonization was analyzed in 11-week-old Arabidopsis plants after 9 weeks of co-cultivation with *R. irregularis* supported by host plants of *Medicago truncatula* in bi-compartmented microcosm systems (Supplementary Fig S1). **a** Percentage of root length colonized by epiphytic and endophytic hyphae of *R. irregularis* in the Arabidopsis mutants *myb72*, *bglu42*, and *f6'h1*, overexpression lines *35S:MYB72* and *35S:BGLU42* and their wild-type Col-0. Percentage of root length colonized by epiphytic and endophytic hyphae of *R. irregularis* in the Arabidopsis overexpression lines *35S:MYB72* and *35S:BGLU42* and Col-0 plants grown with a short photoperiod of 10 h (**b**) and a long photoperiod of 16 h (**c**). Asterisks indicate statistically significant differences compared with Col-0 (mixed-effects models; *, ** and ***correspond to $P < 0.05$, 0.01 and 0.001, respectively). Mean + SE, $n = 4$ for (**a**) and $n = 8$ for (**b**) and (**c**). **d**–**f** Typical colonization phenotype of trypan blue-stained hyphae of *R. irregularis* in *35S:BGLU42* roots. The aseptate epiphytic hyphae (Epi) proliferated vigorously along the surface of *BGLU42*-overexpressing roots, occasionally branched and formed anastomosis (Ana), and developed repeated entry (Entry) attempts. These attempts eventually resulted in fungal penetration into the root, followed by intraradical proliferation by relatively thinner endophytic hyphae (Endo), which often became septate (Sept). Bars = 50 µm

epiphytic hyphae in *35S:BGLU42* was also apparently, albeit non-significantly, increased compared with that of Col-0 ($P = 0.119$; Fig. 2a). Hence, at this point three lines of evidence suggest a positive role of coumarins in the colonization of nonhost Arabidopsis roots by *R. irregularis*: (1) coumarin-secreting Col-0 plants show an enhanced abundance of *Rhizophagus* sp. in its root-associated metagenome (Stringlis et al. 2018b); (2) coumarin biosynthesis mutant *myb72* conditionally shows reduced colonization by host-nursed *R. irregularis* (significant effect in Fig. 1a but not in Fig. 2a); and (3) *MYB72* and *BGLU42*-overexpressing Arabidopsis lines show signs of enhanced colonization by host-nursed *R. irregularis* (Fig. 2a).

MYB72 or BGLU42 overexpression can mitigate AM incompatibility

To obtain more robust evidence for a role of coumarins in AM hyphal colonization of nonhost Arabidopsis roots, we tested whether overexpression of *BGLU42* and of *MYB72* can indeed stimulate the proliferation of the AM fungus in Arabidopsis roots. To this end, we conducted two additional bi-compartmented microcosm experiments with a higher replication level. Because light availability can affect the levels of AM colonization (Johnson et al. 2015), one experiment was conducted with a 10-h photoperiod (Exp. 3) and the other with a 16-h photoperiod (Exp. 4). As expected,

in both Exp. 3 and 4 the roots of *M. truncatula* were heavily colonized by *R. irregularis* (Fig. S2c, d). Furthermore, the neighboring Arabidopsis plants grown under short-day conditions remained in the vegetative state, while the Arabidopsis plants grown under long-day conditions were flowering and produced seeds (not shown). Independently of the photoperiod, the percentage of root length colonized by endophytic hyphae in *35S:MYB72* and *35S:BGLU42* was significantly increased compared with that of Col-0 (Fig. 2b, c), with mean values similar to those observed in Exp. 2 (Fig. 2a) but with higher significance levels, possible due to the higher replication level used in Exp. 3 and 4. Moreover, the morphological development of *R. irregularis* in *35S:BGLU42* was characterized by a vigorous proliferation of aseptate hyphae attached to the root surface with repeated entry attempts (Fig. 2d–f). These epiphytic hyphae sometimes branched and occasionally formed anastomoses between branches (Fig. 2d). However, like in Col-0, once the fungus invaded the root cortex of *35S:BGLU42*, the endophytic hyphae became thinner, had limited proliferation, and often became septate (Fig. 2d).

In order to understand whether the increased susceptibility to AM fungal colonization in *35S:MYB72* and *35S:BGLU42* requires nursery by neighboring host roots, we established in vivo pot experiments in short-day (Exp. 7) and long-day conditions (Exp. 8) with either Arabidopsis or *M. truncatula* plants individually inoculated with *R. irregularis*. As expected, the control roots of individually grown *M. truncatula* plants were heavily colonized (Fig S2e). Similar to what was observed in Exp. 3 and 4, Arabidopsis plants grown in pots under short-day conditions remained in the vegetative state, while plants in pots grown under long-day conditions were flowering and produced seeds (Fig. 3a, b). The colonization of AM hyphae on and in the roots of the tested Arabidopsis lines was strongly reduced in both monoculture pot experiments (Fig. 3c, d) compared with that of the bi-compartmented microcosm experiments (Fig. 2b, c), indicating that host-nursed *R. irregularis* is better capable of colonizing nonhost Arabidopsis roots than AM hyphae that are not growing in a host-supported fungal network. However, despite this reduction, the percentage of root length colonized by the epiphytic hyphae was significantly increased in *35S:BGLU42* grown under short day compared with that of Col-0 (Fig. 3c), while that of *35S:BGLU42* grown in long day was apparently increased, albeit non-significantly (Fig. 3d). The percentage of root length colonized by epiphytic or endophytic hyphae in *35S:MYB72* was not significantly different from that of Col-0 in both pot experiments (Fig. 3c, d), pointing to a weaker ability of this line to stimulate the AM fungus under these growth conditions. No signs of AM fungal colonization were detected in the roots of Arabidopsis and *M. truncatula* plants grown in pots without *R. irregularis* inoculation in Exp. 7 and 8 (data not shown). Collectively, these results suggest that overexpression of *BGLU42*, and to some extend also of *MYB72*, can partially mitigate the mycorrhizal incompatibility of Arabidopsis by stimulating *R. irregularis* asymbiotic colonization in nonhost roots. However, the strength of this mitigation was dependent on nursery of the AM fungal network by neighboring *M. truncatula* host roots.

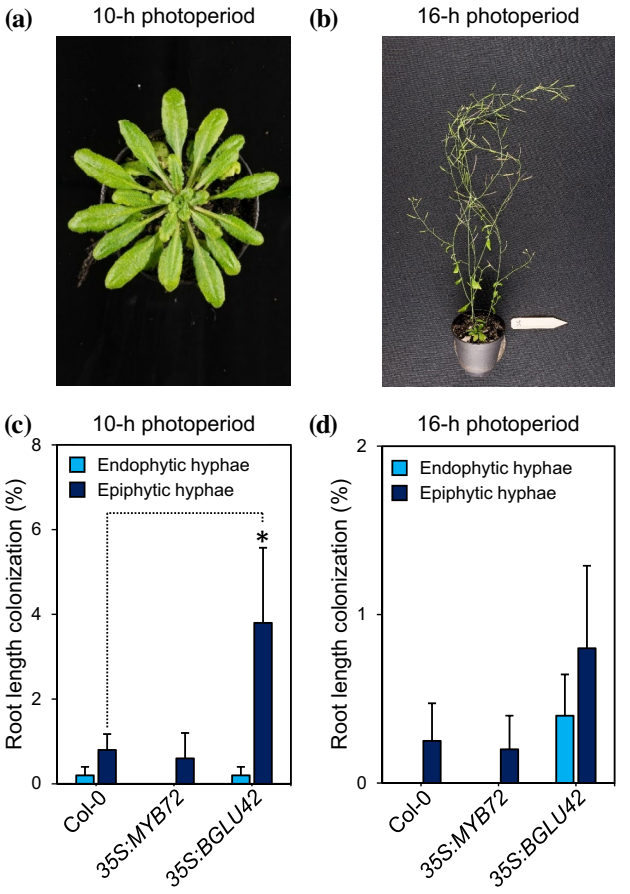

Fig. 3 Colonization by *Rhizophagus irregularis* in nonhost roots of Arabidopsis plants grown in monoculture in pots. The typical aboveground phenotype of Arabidopsis plants grown in a short photoperiod of 10 h (**a**) and a long photoperiod of 10 h (**b**) in pots is shown. Data show the percentage of root length colonized by epiphytic and endophytic hyphae of *R. irregularis* in Col-0, *35S:MYB72*, or *35S:BGLU42* grown under short (**c**) or long (**d**) photoperiod. Colonization was analyzed in 11-week-old Arabidopsis plants grown singly in pots containing inoculum of *R. irregularis* without the nursery by host plants. Asterisks indicate statistically significant differences compared with Col-0 (Duncan's test, $P < 0.05$). Mean + SE, $n = 5$

Overexpression of BGLU42 enhances metabolic activity in *R. irregularis* on Arabidopsis roots

To investigate whether overexpression of *MYB72* or *BGLU42* impacts the physiology of *R. irregularis*, we analyzed SDH-activity inside mycelium networks interacting

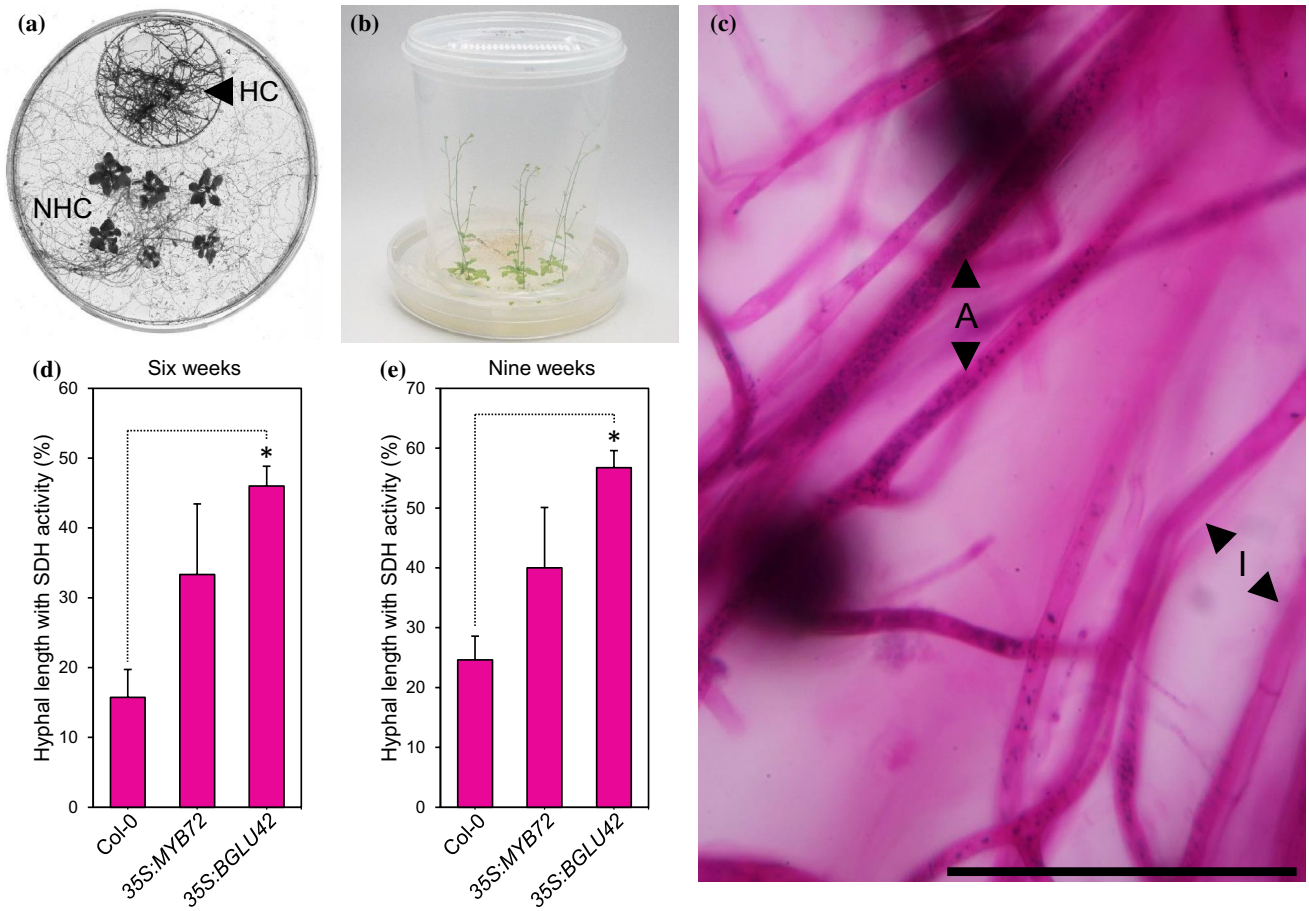

Fig. 4 Metabolic activity of *Rhizophagus irregularis* exposed to Arabidopsis roots. An in vitro bi-compartmented plate system was used to expose the extraradical mycelium of *R. irregularis* to Arabidopsis roots. The system consisted of a lid of a small Petri dish inside a larger Petri dish to create a nonhost compartment (NHC) inside a host compartment (HC), respectively (**a**). The host compartment contained Ri T-DNA-transformed roots of *Daucus carota* to support the growth of *R. irregularis* into the nonhost compartment. Each bi-compartmented plate system was covered with an adapted round microbox container to allow free development of the aerial parts of Arabidopsis plants (**b**). **c** Typical phenotype of hyphae in the extraradical mycelium of *R. irregularis* stained with nitro blue tetrazolium chloride and counter-stained with acid fuchsin to distinguish the metabolic active (A) hyphae, containing blue-black-stained succinate dehydrogenase (SDH) precipitates that mark for mitochondria, from the inactive (I) hyphae stained pink. Bar = 50 µm. Data show the percentage of hyphal length in the extraradical mycelium with SDH activity after 6 (**d**) and 9 (**e**) weeks of fungal exposure to nonhost roots of Arabidopsis Col-0, *35S:MYB72* or *35S:BGLU42*. Asterisks indicate statistically significant differences compared with Col-0 (Duncan's test, $P < 0.05$). Mean + SE, $n = 4$

with Arabidopsis roots in in vitro bi-compartmented growth assays in which the *R. irregularis* mycorrhizal network was nursed by *D. carota* roots (Fig. 4a, b). The staining of SDH, an enzyme complex located in the inner mitochondrial membrane, is a marker for metabolic activity in AM fungi (Saito et al. 1993; Fig. 4c). We conducted two in vitro bi-compartmented plate experiments, in which the *D. carota*-supported AM fungal mycelium was exposed to Arabidopsis plants for 6 (Exp. 9) or 9 weeks (Exp. 10), respectively. Regardless of the time of exposure, in both experiments the roots of *35S:BGLU42* enhanced significantly the percentage of hyphal length with SDH activity compared with that of Col-0 (Fig. 4d, e), while *35S:MYB72* roots apparently increased this parameter, albeit non-significantly (Fig. 4d, e). To understand whether this increase in SDH activity was associated with a larger fungal network, we determined the hyphal length density of the mycelium network 9 weeks after exposure to Col-0, *35S:MYB72* or *35S:BGLU42* (Exp. 10), but found no differences between treatments (Fig. S3). Overall, our results indicate that overexpression of *BGLU42* stimulates the metabolic activity of the extraradical AM mycelium of *R. irregularis* on nonhost roots.

Scopoletin stimulates de novo hyphal elongation and metabolic activity in a dose-dependent manner

BGLU42 activity in Arabidopsis is important for the deglycosylation of the coumarin scopolin and subsequent secretion of its aglycone scopoletin into the rhizosphere (Stringlis et al. 2018b). To test whether scopoletin functions in the chemical communication between Arabidopsis and *R. irregularis*, we exposed germinating spores of *R. irregularis* to scopoletin at concentrations ranging from 2000 to 0.00002 µg mL^{-1} and determined the length and number of newly formed hyphal tips (Exp. 12). We uncovered that scopoletin has a dose-dependent effect on de novo hyphal elongation, with significant stimulatory effects at concentrations 0.2 and 20 µg mL^{-1} 5 days after application (Fig. 5a, b, c, e), while at earlier stages only a trend for a positive effect by 0.2 µg mL^{-1} concentration was observed (Fig. 5a). High concentrations of scopoletin (2000 µg mL^{-1}) were toxic for *R. irregularis*. In a second experiment (Exp. 13), we confirmed the dose-dependent effect of scopoletin on hyphal elongation with a significant stimulation of hyphal length at 7 days after application of 0.2 µg mL^{-1} of scopoletin concentration (Fig. S4a). In contrast to the impact on hyphal elongation, scopoletin did not affected the number of newly formed hyphal tips, used here as a proxy for hyphal branching (Figs. 5b–e, and S4b).

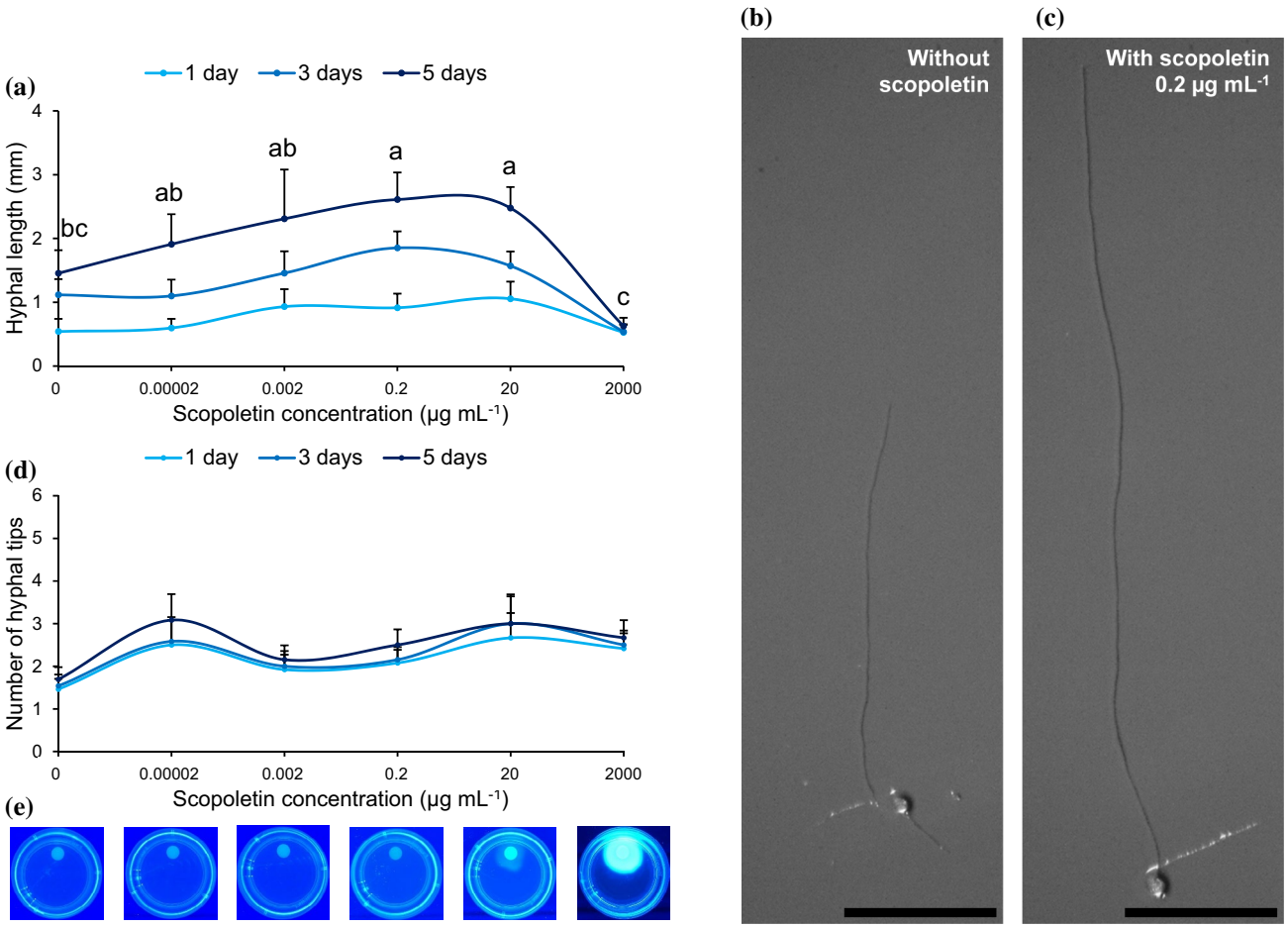

Fig. 5 Impact of scopoletin on germinating spores of *Rhizophagus irregularis*. The paper disk diffusion test was used to expose *R. irregularis* spores to scopoletin solutions with concentrations varying from 0.00002 to 2000 µg mL^{-1} in purged 70% ethanol. Purged 70% ethanol was used as the mock control without scopoletin (0 µg mL^{-1}). Data show a time-course study of the length (**a**) and the number of tips (**d**) of hyphae germinating from a single spore of *R. irregularis*, at 1, 3, and 5 days after scopoletin application. For each time point in each chart, mean values with similar letters or without letters are not significantly different among each other (Duncan's test, $P < 0.05$). Mean + SE, $n = 13$. The phenotype of a 6-day-old germinated single spore of *R. irregularis* treated with purged 70% ethanol mock control without scopoletin (**b**) or with 0.2 µg mL^{-1} of scopoletin (**c**) 5 days after the application is shown. Bars = 500 µm. **e** Confirmation under UV light of fluorescent activity of scopoletin in the step-wise diluted concentrations and mock control used in the paper disk diffusion test

To understand whether scopoletin can stimulate the metabolic activity of *R. irregularis*, we allowed *D. carota*-nursed AM fungal networks to re-grow on a medium containing either 0, 0.5 or 7.7 µg mL^{-1} of scopoletin (Exp. 11) in an in vitro bi-compartmented plate system (Fig. S5a). After a growth period of 8 weeks (please note that *R. irregularis* grows very slow in this system), we found a dose-dependent effect of scopoletin on fungal metabolic activity, with a significant stimulatory effect of scopoletin at 0.5 µg mL^{-1} concentration (Fig. S5b). The hyphal length density was also higher in the scopoletin-exposed mycelium, but this was not statistically significant (Fig. S5c). Overall, our results suggest that scopoletin can have a positive effect on AM fungal growth and metabolism.

Impairment of defense and growth hormone signaling did not influence AM incompatibility nor growth antagonism

In a previous RNA-seq study, we compared *R. irregularis*-mediated changes in root gene expression in host *M. truncatula* and nonhost Arabidopsis roots growing in the same AM network (Fernández et al. 2019). We found that, in contrast to compatible roots of *M. truncatula*, *R. irregularis*-mediated changes in gene expression in Arabidopsis roots were enriched for genes related to SA-dependent defenses and glucosinolate-related defense responses that are typically regulated by JA/ET-dependent signaling (Pangesti et al. 2016). To investigate the role of SA- and JA/ET-mediated defense signaling in the colonization of Arabidopsis roots by host-nursed AM fungi, we tested the SA signaling mutants *sid2*, *eds5* and *npr1,* and the JA-/ET signaling mutants *dde2* and *ein2* in the bi-compartmented microcosm setup (Exp. 5). In mutants *sid2, npr1, dde2,* and *ein2*, the percentage of root length colonized by endophytic hyphae was similar to that observed in Col-0 (Fig. S6a). Mutant *eds5* had a minor but significant ($P < 0.050$) increase in the percentage of root length colonized by the endophytic hyphae (Fig. S6a), with a mean value still at least tenfold lower than that in *M. truncatula* roots (Fig. S2f). Together, this indicates that, if any, SA- and JA/ET-dependent defenses have a negligible role as a first-line mechanism of mycorrhizal incompatibility. Additionally, in all mutants tested in Exp. 5, the percentage of root length colonized by epiphytic hyphae was not significantly different from that observed in Col-0 (Fig. S6a), indicating that SA- and JA/ET-dependent defense pathways do not have a major influence on the proliferation of the host-nursed AM fungus along the nonhost root surface.

Colonization of Arabidopsis roots by host-nursed *R. irregularis* is associated with a significant repression of nonhost plant growth (Veiga et al. 2013; Fernandez et al. 2019). To understand the role of defense or growth hormone pathways in this process, we compared the shoot mycorrhizal growth response of the defense hormone-related mutants *sid2*, *eds5*, *npr1*, *dde2*, and *ein2* (Exp. 5), and of the Arabidopsis genotypes compromised in the growth hormone signaling pathways of auxin (*tir1afb2afb3*), BR (*rot3*), CK (*P10:CKX3* and *35S:CKX3*) or GA (*ga20ox1ga20ox2*) (Exp. 6) with that of Col-0. As expected, Col-0 plants displayed a negative aboveground mycorrhizal growth response upon colonization of the roots by *M. truncatula*-nursed *R. irregularis* (Figs. S6a, b and S7a, b). The aboveground growth of the host plant *M. truncatula* was somewhat variable (Fig. S8a, b) but was not significantly affected by *R. irregularis* colonization ($P = 0.150$; Fig. S2f, g), with an average mycorrhizal growth response of $2.5 \pm 3.0\%$ across all treatments of Exp. 5 and 6 (Mean ± SE, $n = 24$). A similar pattern was observed in Exp. 1 and 2 ($P = 0.210$; Figs. S2a, b; S8c, d). The aboveground mycorrhizal growth response of *sid2*, *eds5* and *npr1* was negative and not significantly different from that of Col-0 (Fig. S7a). The same holds true for the mutants *dde2* and *ein2* (Fig. S7a). Together, this indicates that defenses regulated by the SA and the JA/ET pathway are not likely to significantly contribute to the observed *R. irregularis*-mediated growth repression in Arabidopsis. Similarly, all growth hormone-related Arabidopsis genotypes colonized by *R. irregularis* (Fig. S6b), i.e. *tir1afb2afb3*, *rot3*, *35S:CKX3*, *P10:CKX3*, and *ga20ox1ga20ox2*, showed negative shoot mycorrhizal growth responses that were not significantly different compared with that of Col-0 (Fig. S7b), albeit that *P10:CKX3* displayed a marginally non-significant trend ($P = 0.053$) for an even greater *R. irregularis*-mediated growth reduction (Fig. S7b). Also, the nonhost defense and coumarin-related Arabidopsis genotypes did not provide evidence for a role of these factors in mycorrhiza-mediated growth repression (Fig. S7c, d). Altogether, our data indicate that the tested defense and growth hormone pathways do not seem to operate as a first-line mechanism of mycorrhizal incompatibility, and neither do they affect *R. irregularis*-mediated growth suppression in Arabidopsis.

Discussion

Symbiotic incompatibility between land plants and AM fungi evolved many times during plant evolution (Wang and Qiu 2006; Brundrett and Tedersoo 2018), but the molecular mechanisms influencing the inability of AM fungi to form intimate associations with nonhost roots are not completely understood (Cosme et al. 2018). The development of AM symbiosis unfolds along a sequence of fungal colonization steps that culminate with an intimate formation of morphological markers of symbiotic exchange inside the root cortex cells, i.e. arbuscules, hyphal coils and/or arbusculated coils. First, a mutual recognition of pre-penetration signals in the rhizosphere entails the secretion of root SL and fungal

lipochitooligosaccharides and short-chain chitin oligomers (Akiyama et al. 2005; Maillet et al. 2011; Genre et al. 2013). Then, physical attachment of the fungus to the root surface is followed by the development of entry points for penetration of the root by the fungal hyphae. Several symbiotic genes characterized in legumes, such as *NFP*, *DMI2*, *DMI3*, *CASTOR*, and *IPD3*, which stimulate early steps of AM colonization (Delaux et al. 2013), are absent from the genome of Arabidopsis (Delaux et al. 2014). However, our study together with previous reports (Veiga et al. 2013; Fernández et al. 2019) show that, when Arabidopsis plants grow in conditions that simulate the natural process in which early plant growth occurs side-by-side with host plants in the presence of a pre-established, host-nursed AM mycelium (Francis and Read 1994; Lambers et al. 2018), the AM fungi can attach to the surface of the nonhost roots, form hyphopodia-like structures, penetrate into the root, and proliferate intraradically to some extent. Moreover, the upregulation of Arabidopsis SL biosynthesis genes *CCD7* and *CCD8* in response to *R. irregularis* indicates that nonhost roots can specifically detect AM fungi (Fernández et al. 2019). Altogether, this suggests that early steps of AM colonization are not blocked in Arabidopsis plants. The present study shows that Arabidopsis *MYB72* and *BGLU42* overexpression can promote AM colonization in nonhost roots, which indicates that additional factors, besides those encoded by the canonical symbiotic toolkit genes present in host genomes (Delaux et al. 2014), influence the early steps of AM colonization. In particular, when the fungus was nurtured by neighboring host plants, *BGLU42* overexpression led to a vigorous proliferation of epiphytic hyphae along the surface of Arabidopsis roots with repeated entry attempts, mostly between epidermal root cells. This entry mode also occurs in host roots depending on the plant species (Dickson 2004). In our study, fungal entry attempts eventually resulted in successful penetration into the nonhost roots. Consequently, *BGLU42*-overexpressing roots harbored higher levels of intraradical colonization by endophytic hyphae compared with Col-0 roots. However, these endophytic hyphae often became septate, which indicates retraction of fungal cytoplasm and senescence (Cosme et al. 2018). Moreover, the fungal invasion of *BGLU42*-overexpressing roots was associated with enhanced metabolic activity of the AM mycelium outside the roots but did not result in formation of morphological markers of symbiotic exchange, indicating a predominantly endophytic incompatibility. In contrast to *BGLU42* overexpression, *MYB72* overexpression stimulated endophytic colonization without affecting epiphytic colonization, possibly due to their specific molecular functions. Although *BGLU42* is co-regulated by MYB72, *MYB72* overexpression does not constitutively induce *BGLU42* (Zamioudis et al. 2014). Instead, *MYB72* overexpression constitutively induces key genes of the phenylpropanoid pathway, including genes regulating coumarin biosynthesis, and genes belonging to families that contain members of the symbiotic toolkit known to impact endophytic colonization by AM fungi in model host legumes, such as phosphate and ABC transporter genes (Javot et al. 2007; Zhang et al. 2010; Zamioudis et al. 2014; Bravo et al. 2017). In our study, the strength of the effects of *MYB72* or *BGLU42* overexpression on fungal colonization were nonetheless conditioned by the presence of neighboring host plants, which might be explained by the biotrophic dependency of *R. irregularis* on host-supplied lipids promoted by plant orthologues of the symbiotic toolkit that is absent in nonhosts such as Arabidopsis (Delaux et al. 2014; Bravo et al. 2017). Overall, our results suggest that mycorrhizal incompatibility is largely manifested at a late stage of fungal colonization, during hyphal proliferation within nonhost roots, which sheds new light on the molecular mechanisms preventing AM fungi from forming intimate associations with the roots of nonhost plants.

MYB72-dependent BGLU42 activity is important for the deglycosylation of the coumarin scopolin and subsequent secretion of its aglycone scopoletin from the root interior into the rhizosphere (Stringlis et al. 2018b). We are not aware of other documented targets of BGLU42, hence we focused on the putative role of the major coumarin scopoletin in the interaction between *R. irregularis* and Arabidopsis. In our study, we employed the paper disk diffusion system similar to the one previously used to establish SLs as pre-penetration signals in host plants (Akiyama et al. 2005) and used more recently to establish the stimulation of asymbiotic sporulation in AM fungi by fatty acids (Kameoka et al. 2019). By using this system, we uncovered that scopoletin stimulates the elongation of hyphae germinating from single spores of *R. irregularis* in a dose-dependent manner, and independently from the presence of plant roots. Moreover, we showed that scopoletin can stimulate the metabolic activity in extraradical mycelium of *R. irregularis*. Taken together, this indicates that scopoletin may act as a plant signal positively affecting pre-penetration steps of asymbiotic AM colonization in the nonhost Arabidopsis, and might be the primary mechanism by which *BGLU42* overexpression stimulates fungal metabolism and colonization in Arabidopsis. Whether in vivo scopoletin application to Col-0 can mimic the colonization phenotype of *35S:BGLU42* remains to be elucidated. Scopoletin is a simple coumarin produced in many plant species, including compatible hosts for AM fungi, such as *Solanum lycopersicum* and *M. truncatula*, among others (Wang and Qiu 2006; Stringlis et al. 2019). In Arabidopsis, the coumarin scopoletin is produced via the phenylpropanoid pathway (Stringlis et al. 2018b). Recent studies in Arabidopsis have uncovered that root-secreted coumarins play an important role in Fe acquisition (Tsai and Schmidt 2017a; Stringlis et al. 2018b, 2019), and are probably the Fe-mobilizing phenolic compounds released by compatible host roots of *Trifolium pratense* to improve mobilization of scarcely

available Fe (Wang and Qiu 2006; Jin et al. 2007; Stringlis et al. 2019). Similar to SL, scopoletin and other coumarins are also secreted by roots under low Pi conditions (Mayzlish-Gati et al. 2012; Ziegler et al. 2016), possibly as part of an orchestrated plant effort to facilitate Fe acquisition and prevent Fe deficiency-mediated arrest of root architectural responses required to improve the direct pathway for Pi uptake (Tsai and Schmidt 2017b). This, together with our results, suggests that Arabidopsis harbors Pi and Fe-regulated signals capable of stimulating colonization by AM fungi, although colonization in nonhost roots is largely dependent on nursery by neighboring host plants. In Arabidopsis roots, application of the SL analogue GR24 induces a MAX2-dependent accumulation of the PAL1 enzyme that catalyzes the first step of the phenylpropanoid pathway (Walton et al. 2016). Interestingly, impairments in the phenylpropanoid pathway reduce colonization by the ectomycorrhizal fungus *Laccarcia bicolor* in host plants of poplar (Behr et al. 2020). Altogether, this points to the existence of an intricate relationship among Pi and Fe availability in the soil environment and the root production of SL and phenylpropanoid compounds, with potential implications for different plant–mycorrhizal fungus associations.

In this study, we also aimed to test the hypotheses that plant hormone pathways can influence fungal colonization in nonhost roots and that the growth repression observed in nonhost plants after colonization of the roots by host-supported AM fungi is caused by growth-defense tradeoffs. However, the fungal colonization and the negative mycorrhizal growth response was in all defense and growth hormone-related mutants and overexpressors genotypes mostly similar to that observed in wild-type Col-0 plants. Whether combinations of these pathways or even different pathways are involved remains to be elucidated. We, thus, concluded that none of tested defense- or growth-related processes alone operate as a first-line mechanism of mycorrhizal incompatibility nor have a significant impact on the mycorrhiza-mediated growth repression observed in nonhost plants.

In summary, this study sheds new light on the biological mechanisms involved in mycorrhizal incompatibility in nonhost plants. We provide evidence that plant-derived coumarins can mitigate mycorrhizal incompatibility in nonhost Arabidopsis plants. In this context, the coumarin scopoletin emerged as a novel signal capable of stimulating AM fungi in the prepenetration phase of the plant–AM fungus interaction.

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Author contributions All authors planned and designed the research. MC performed the experiments. MC, SD, MGAvdH and CMJP analyzed results. All authors wrote the manuscript.

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