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Indolic glucosinolate pathway provides resistance to mycorrhizal fungal colonization in a non-host Brassicaceae

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Abstract. Most terrestrial plants form mycorrhizas, but a number of agricultural plants, including the Brassicaceae, are non-mycorrhizal. Brassicaceae can still be colonized by arbuscular mycorrhizal fungi (AMF), but species like *Arabidopsis thaliana* experience growth reductions following AMF colonization at similar magnitude to that of fungal pathogen infections and lack key genes necessary for nutrient exchange. *Arabidopsis* also produces specific secondary compounds via the modification of tryptophan, including indolic glucosinolates (IGs), which have anti-fungal properties and may therefore be involved in reducing AMF colonization. This study therefore addressed whether the ability to produce IGs facilitates resistance to AMF colonization and growth suppression. We challenged with AMF inoculation transgenic *Arabidopsis* lines which produce no or enhanced IGs levels in comparison with the wild-type. Arbuscular mycorrhizal fungal inoculation suppressed the development of IG-removed plants, activated their pathogen-response defenses, and enhanced AMF vesicle colonization of their root systems. Arbuscular mycorrhizal fungi had no detrimental effects on wild-type or IG-enhanced plants. Using BLAST to identify IG orthologs across 29 Brassicales, we also show that non-mycorrhizal species possess orthologous proteins for IG biosynthesis to *Arabidopsis* which AMF-associated Brassicales lack. In conclusion, the IG production pathway appears to serve an important and previously unknown role in reducing AMF colonization in *Arabidopsis* and may serve similar functions in non-host Brassicales more broadly.

Key words: *Arabidopsis*; arbuscular mycorrhizal fungi; Brassicaceae; glucosinolates; indolic glucosinolates.

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INTRODUCTION

Few angiosperms do not form symbiotic relationships with arbuscular mycorrhizal fungi (AMF; Brundrett and Tedersoo 2018). The large and agriculturally important plant family Brassicaceae forms what have been called rudimentary arbuscular mycorrhizal (RAM) phenotypes (Cosme et al. 2018) because members of this family are occasionally colonized by AMF (Tommerup 1984, Regvar et al. 2003, Veiga et al. 2013, Fernández et al. 2019). However, AMF colonization in these plants is not accompanied by the breadth of exchanges which comprise a bona fide mycorrhiza (Cosme et al. 2018). Notably, colonization is low, and arbuscules, the primary structures which facilitate nutrient exchange, are seldom formed on their roots systems (but see Hirrel et al. 1978, Tommerup 1984, Regvar et al. 2003). Brassicaceae also lack symbiosis genes for phosphorus and nitrogen transport (Delaux et al. 2014), and there is no evidence they benefit nutritionally from AMF (Cosme et al. 2018, Fernández et al. 2019). Thus, despite a

RAM phenotype, the Brassicaceae are traditionally considered non-mycorrhizal (Delaux et al. 2014, Brundrett and Tedersoo 2018). Brassicaceae also evolved from a true mycorrhizal ancestor meaning new ecological opportunities selected against symbiosis (Vigneron et al. 2018). Arbuscular mycorrhizal fungal colonization can reduce Brassicaceae growth at similar magnitudes to that of fungal pathogens (Veiga et al. 2013, Fernández et al. 2019). It is therefore possible that the Brassicaceae evolved strategies which actively reduce interactions with AMF to counter AMF-induced growth suppression, but this remains unknown.

One way Brassicaceae may reduce AMF colonization is through the production of secondary chemicals known as glucosinolates. Glucosinolates are a broad class of secondary chemicals produced by the Brassicaceae and some other plants in the Brassicales (order; Fahey et al. 2001). Glucosinolates are toxic to fungal pathogens (Zhang et al. 2015), endophytes (Nongbri et al. 2012), as well as AMF when added to growing media or applied to the roots of arbuscular mycorrhizal plants (Stinson et al. 2006, Cantor et al. 2011); however, whether these chemicals also function to reduce AMF colonization on non-mycorrhizal or RAM (hereafter non-mycorrhizal) Brassicaceae roots remains unclear. Since some members of the Brassicales form functioning arbuscular mycorrhizas and also produce glucosinolates (Hirrel et al. 1978, Vierheilig et al. 2000, Cosme et al. 2018), the hypothesis that glucosinolates are involved in suppressing AMF colonization has been discarded (Cosme et al. 2018). However, the quantity and chemical diversity of glucosinolates produced by Brassicaceae may be different between arbuscular mycorrhizal and non-mycorrhizal members of the Brassicales (Vierheilig et al. 2000, Fahey et al. 2001).

In particular, indolic glucosinolates (IGs) produced via the conversion of tryptophan are not synthesized by most mycorrhizal-associated plant families which produce other glucosinolates, but they are present in plants across the Brassicaceae (Fahey et al. 2001, Mithen et al. 2010). Even within plant families that are both mycorrhizal-forming and IG producing, IGs are not produced at significant levels (Ludwig-Müller et al. 2002) and the chemical diversity of IGs is low in these plants (Vierheilig et al. 2000, Fahey et al. 2001). Whether AMF host plants which produce IGs possess similar IG encoding proteins as non-mycorrhizal species has not been determined, though this would provide insight into their potential role in reducing AMF colonization. Since IGs are activated alongside various plant defense systems (Clay et al. 2009), and unlike other glucosinolates, IGs are not volatile (Zukalova and Vasak 2002), this may preserve IG concentrations in the root and inhibit AMF colonization and development. Here, we specifically examined the role of IGs in reducing AMF colonization in *Arabidopsis thaliana* (Brassicaceae) using transgenic plant lines which either produce no or higher levels of IGs compared to the wild-type.

Arabidopsis has a well-annotated IG biosynthesis pathway (Sønderbyet al. 2010), and transgenic lines have been developed with either no or enhanced capacity to produce IGs (Zhao et al. 2002, Celenzaet al. 2005), making this an ideal model system to test the role of IGs in deterring AMF symbiosis using a novel, direct experimental approach. We inoculated soils of transgenic *Arabidopsis* (IG-removed or IG-enhanced) and the Col-0 accession (hereafter wild-type) with *Rhizophagus irregularis*, a common, generalist AMF species that has been previously shown to colonize *Arabidopsis* (Veiga et al. 2013, Fernández et al. 2019). We then measured plant biological and reproductive fitness, salicylic acid contents, and AMF colonization. Salicylic acid is a key hormone which guides the expression of induced plant defenses (Dodds and Rathjen 2010), and if *Arabidopsis* can produce IGs which deter AMF colonization, then modified plants lacking IG production capacity should have higher salicylic acid levels relative to plants which produce IGs. We hypothesized that (1) AMF inoculation would reduce plant biological and reproductive fitness (IG-removed > wild-type > IG-enhanced plants) since plants lacking IG cannot counter AMF-induced growth suppression; (2) AMF inoculation would increase salicylic acid levels (IG-removed > wild-type > IG-enhanced plants); and (3) AMF colonization would be highest in IG-removed followed by wild-type and then IG-enhanced plants under the assumption that IGs are directly involved in reducing AMF colonization.

Methods

Plant strains

This work was conducted using *Arabidopsis* of the Col-0 accession (thereafter referred to as "wild-type") obtained from TAIR (Berardini et al. 2015). We used well-characterized mutants that either do not produce IGs (the cyp79B2 cyp79B3 double mutant referred to as "IG-removed"; Zhao et al. 2002) or which have elevated IG production relative to the wild-type (the atr1D allele of MYB34 hereafter referred to as "IG-enhanced"; Celenza et al. 2005).

AMF spores, growth medium, and plant propagation

Spores of *R. irregularis* (culture-line DAOM197198, DAOM181602, and MUCL43194) were obtained from Symplanta (Graupnerweg, Germany). The inoculum contained 1 million spores in 250 g of food-grade diatomaceous earth. Growing medium was prepared using sterilized (autoclaved 2× for 36 min at 121°C) Miracle-Gro Premium Potting Mix (Marysville, Ohio, USA) amended with AMF spores. We added AMF at two realistic levels naturally observed in field studies for a total of three AMF spore levels: 0, 10, and 50 spores per gram growth medium (Sieverding et al. 1989, Picone, 2000, Stürmer and Siqueira 2011, Yang et al. 2011, Aleixo et al. 2014). To make growing media equivalent, we also added sterilized, food-grade diatomaceous earth to the 0 and 10 spores per gram levels treatments,

Plants were grown in individual plastic pots (5 × 5 cm) with 100 g of growth medium at 25°C with 50% relative humidity under 10,000 lumen fluorescent lamps on a 16:8 hours on: off light cycle. Twenty cold stratified seeds were sown on top of six replicate pots per treatment (3 genotypes × 3 AMF levels × 6 replicates = 54 sampling units). We allowed multiple seeds to germinate until a single plant produced true leaves, after which all remaining plants were removed to maintain one plant per pot. Plants were bottom watered 3–4 times a week with 1–10 mL of ultra-purified deionized water (without nutrients) depending on development stage (mature plants were watered four times a week).

Measuring performance and harvesting plants

We tracked the performance of plants over the course of their development until reaching a set growth stage (i.e., principle growth stage 7; Boyes et al. 2001). The period at which plants reached the principle growth stage varied across treatments ranging between 5 and 7 weeks, but we sampled plants at the same growth stage. Unless plants died early or began to die before producing fruits (siliques), each plant was grown until flowering was complete and siliques began to ripen just prior to shattering. At this point, we measured the number of fruits and height of the plants from the base of the rosette to the highest stem. We then scored the overall biological and reproductive performance of plants based on whether (1) they died early, (2) began to die before producing siliques, (3) began to die immediately after producing siliques, (4) were fit (biological and reproductive) with minimal discoloring/wilting, or (5) were fit with no discoloring/wilting.

We then harvested the plants and split the aboveground and belowground components. A tissue sample from each plant was taken and dried at 60°C for 96 h to calculate moisture content. This dry tissue was then finely ground, and total tissue carbon and nitrogen contents were quantified using dry combustion on a Perkin Elmer 2400 Series II CHN elemental analyzer (Waltham, Massachusetts, USA). Separate leaf samples were taken from each plant. One was immediately flash-frozen in liquid N and stored at −20°C for salicylic acid quantification. The second sample was immediately placed in HPLC grade methanol and stored at −20°C for quantification of glucosinolate and camalexin, a second defense compound produced from an intermediate in the IG pathway (Bender and Celenza 2009). Primary and lateral roots were collected from each plant, rinsed, and used to measure AMF colonization.

Salicylic acid, camalexin, IG, and AMF colonization measurements

We measured salicylic acid contents on leaves using a modified spectrophotometric procedure (Warrier et al. 2013). Frozen leaf tissues were ground in liquid N to a fine powder. Deionized water (1 mL) was added to the ground tissue

(50–200 mg) and vortexed for 45 s and then centrifuged at 10,000 *g* for 10 min. Clear supernatant (9 μL) was added to 1% ferric chloride solution (261 μL) in clear 96-well plates which were then incubated for 5 min at 25°C in the dark. Violet color development was analyzed spectrophotometrically at 540 nm and quantified using a salicylic acid standard curve.

Glucosinolate content was measured on 50–200 mg of fresh leaf tissue suspended in HPLC grade methanol (1 mL; the same extract was used to quantify camalexin after glucosinolate removal by anion exchange chromatography). We measured desulfoglucosinolates using reverse-phase HPLC and methods as described previously (Pieck et al. 2015). Camalexin was quantified from the supernatant after glucosinolates were removed. This supernatant was then lyophilized, resuspended in HPLC grade MeOH, incubated at 37°C for 10 min, vortexed, and prepared for HPLC analysis by filtering with a 0.22-μM PVDF syringe filter. Camalexin was analyzed on a Waters 2795 HPLC (Waters, Milford, Massachusetts, USA) fitted with a reverse-phase C-18 Luna 5 μmol/L, 4.6 × 250 mm column and matching guard column (Phenomenex, Torrance, California, USA). Samples were run over a gradient of 60–100% methanol over 14 min, and the eluent was monitored by diode array detection between 210 and 400 nm (2-nm interval). Camalexin was identified by comparison of retention time and UV absorbance at 318 nm to authentic camalexin (AfferChem, New Brunswick, New Jersey, USA). We quantified the IGs indoly-3-methyl desulfoglucosinolate (I3M), 4-methoxyindol-3-methyl desulfoglucosinolate (4MOI3M), 1-methoxyindol-3-methyl desulfoglucosinolate (1MOI3M), and the aliphatic glucosinolate 4-methylsufinylbutyl glucosinolate (4MSOB) to an I3M standard.

We collected roots to quantify AMF colonization using the grid-intersection method (McGonigle et al. 1990). Roots were cleared at 90°C in 10% KOH solution for 20 min, acidified in 2% HCl for 15 min, and immersed in a 0.03% chlorazol black solution (glycerol, deionized water, and lactic acid; 1:1:1; v:v:v) for 3 h at 90°C. Stained roots were measured for hyphal, arbuscule, and vesicle colonization after first assessing internal structures at 400× magnification and then after 150 passes at 100× magnification on a compound microscope.

Statistics

All statistical analyses were conducted in R (R Core Team 2018), and significance was set at $P \leq 0.05$. We used linear mixed effects models to determine the influence of AMF inoculum density, plant genotype, and their interaction on reproductive output (number of fruits per branch), plant height (cm), aboveground biomass (mg of dry tissue), tissue N content (%), and tissue C content (%). Since there were significant AMF inoculum × genotype interactions for the three developmental responses (i.e., reproductive output, height, aboveground biomass; Appendix S1: Table S1), we calculated the difference between sterile (no AMF inoculum) and AMF-amended plants to compare the effect size of AMF on development across genotypes (Appendix S1: Table S2). We calculated traditional 95% confidence intervals for the effect size using the groupwise Means function in the rcompanion package (Mangiafico and Mangiafico, 2017).

AMF inoculation had a strong impact on the IG-removed plants, and there was insufficient biomass for salicylic acid analysis or AMF colonization on half of these plants, so we combined the 10 and 50 spores/g dry soil treatments and compared these to the sterile soils as a two-level factor analysis (sterile vs. AMF).

We used the lme function in the nlme package (Pinheiro et al. 2017) and replicate as a random variable in all models. Due to heteroscedasticity, we also created models with unequal variance structures and used these models if they significantly lowered the AIC score relative to the base model. Contrasts were made using individual *t*-tests with homoscedastic or heteroscedastic variance to test our main hypotheses, and these results are shown using different letters in the display items to denote significant pair-wise differences. Model residuals were inspected for normality using qqnorm plots and Shapiro-Wilk tests of normality.

Results

IG production capacity of the transgenic plants matched a priori expectations

Total IG contents of each plant genotype across treatments were consistent with expectations. IG-removed plants did not produce detectable IG levels across any treatments, while wild-type

and IG-enhanced plants had 1.6 ± 0.4 (overall mean ± SE) and 14 ± 2.5 nmol IG/mg dry tissue, respectively (Fig. 1). None of the plants produced detectable levels of camalexin across any treatments. The IG production capacity of each plant genotype therefore matched expected IG production capacity. Camalexin, which is also produced via the IG production pathway, was not produced in the plants which allowed us to more directly address our hypotheses. Since Arabidopsis also produces aliphatic glucosinolates (AGs), we quantified the abundant AG 4MSOB to assess whether its production was altered by the genotypes used. 4MSOB was less concentrated in wild-type and IG-enhanced plants compared to IG-removed plants levels with 1 ± 0.3 and 0.8 ± 0.2 nmol/mg dry tissue, respectively. IG-removed plants produced 1.7 ± 0.4 nmol 4MSOB per mg dry tissue.

IG-removed plants had lower fitness, higher salicylic acid contents, and greater AMF vesicle colonization in soil with AMF inoculation compared to wild-type or IG-enhanced plants

AMF inoculation reduced the overall performance (i.e., growth and reproductive fitness) of IG-removed plants compared to those grown in sterile soil without AMF, while the performance of wild-type and IG-enhanced plants was similar under both sterile and AMF-inoculated conditions (Fig. 2). In sterile soil, the performance of all three genotypes was the same. Arbuscular mycorrhizal fungi inoculation caused half of the IG-removed plants to senesce prior to producing fruits. Arbuscular mycorrhizal fungi inoculation also reduced the reproductive output on IG-removed plants by ten fruits per branch but had no effect on wild-type plants while marginally increasing reproductive output on IG-enhanced plants (Fig. 3). Plant height response mirrored reproductive output. Arbuscular mycorrhizal fungal additions reduced aboveground biomass of IG-removed plants by 30–175 mg, though this was only significant at the higher inoculation level (50 spores/g dry soil). Arbuscular mycorrhizal fungi did not affect the biomass of wild-type or IG-enhanced plants. There were also no differences in tissue C or N contents across genotypes or in AMF-inoculated vs. sterile soils (Appendix S1: Table S1).

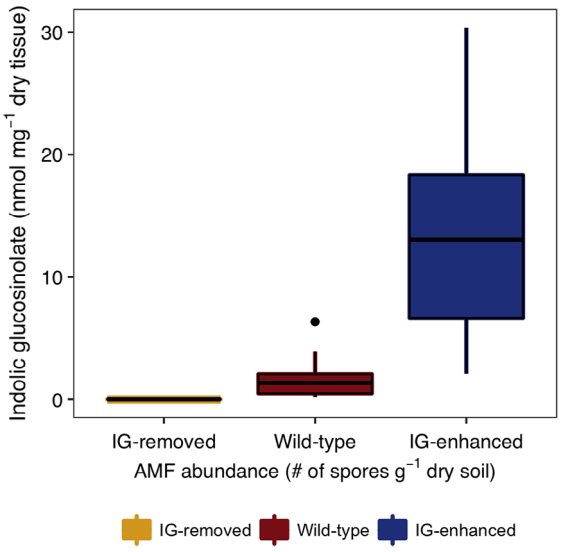

Fig. 1. Indolic glucosinolate concentrations across plant genotypes. Bars represent the interquartile range, whiskers show the minimum and maximum values, points show suspected outliers, and the horizontal black lines show the median.

Arbuscular mycorrhizal fungal inoculation significantly increased salicylic acid content in IG-removed plants but not in wild-type or IG-enhanced plants (Fig. 3). In sterile soil, all three genotypes had the same amount of salicylic acid. Since plant performance of IG-removed plants was significantly reduced with AMF inoculation (Fig. 2), only a subset of the IG-removed plants produced sufficient healthy leaf tissue for destructive sampling ($n = 5$; we prioritized healthy tissue allocation to IG and camalexin quantification); therefore, we bulked IG-removed tissues from both AMF inoculation levels (10 and 50 spores/g dry soil). IG-removed plants produced more salicylic acid when grown in soil with AMF inoculation compared to sterile soil (Fig. 3). Conversely, there was no difference in salicylic acid contents between sterile and AMF-inoculated growth environments for wild-type or IG-enhanced plants.

Arbuscular mycorrhizal fungal colonization was <10% across all plant genotypes, but it was highest on roots of the IG-removed plants and was 99% vesicular (Fig. 4). There were no discernible arbuscules and few intraradical hyphae (<1%) on all root systems. Vesicles were observed for all plant genotypes, but vesicle colonization

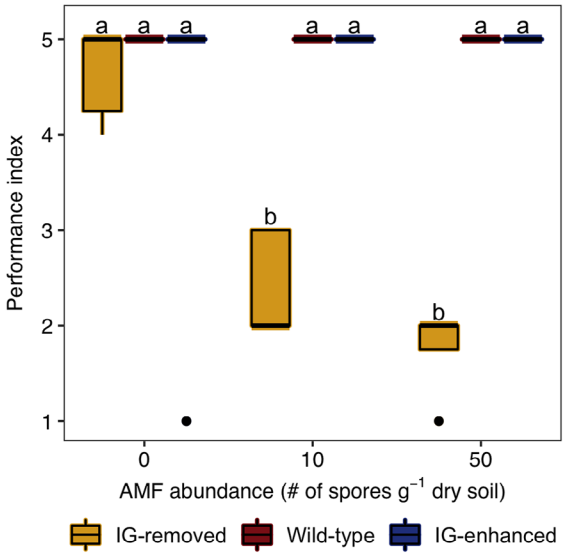

Fig. 2. Performance index across plant genotypes in response to varying arbuscular mycorrhizal fungal inoculation levels. Performance values represent plants that (1) died before bolting, (2) began dying before reproduction, (3)began dying after reproduction, (4) were fit with little discoloring or wilting, and (5) were fit with no discoloring or wilting. Bars represent the interquartile range, whiskers show the minimum and maximum values, points show suspected outliers, and the horizontal black lines show the median. The wild-type and enhanced plants had the same value (5) across all replicates minus one suspected outlier. Bars with different letters are significantly different ($P \leq 0.05$) based on independent contrasts.

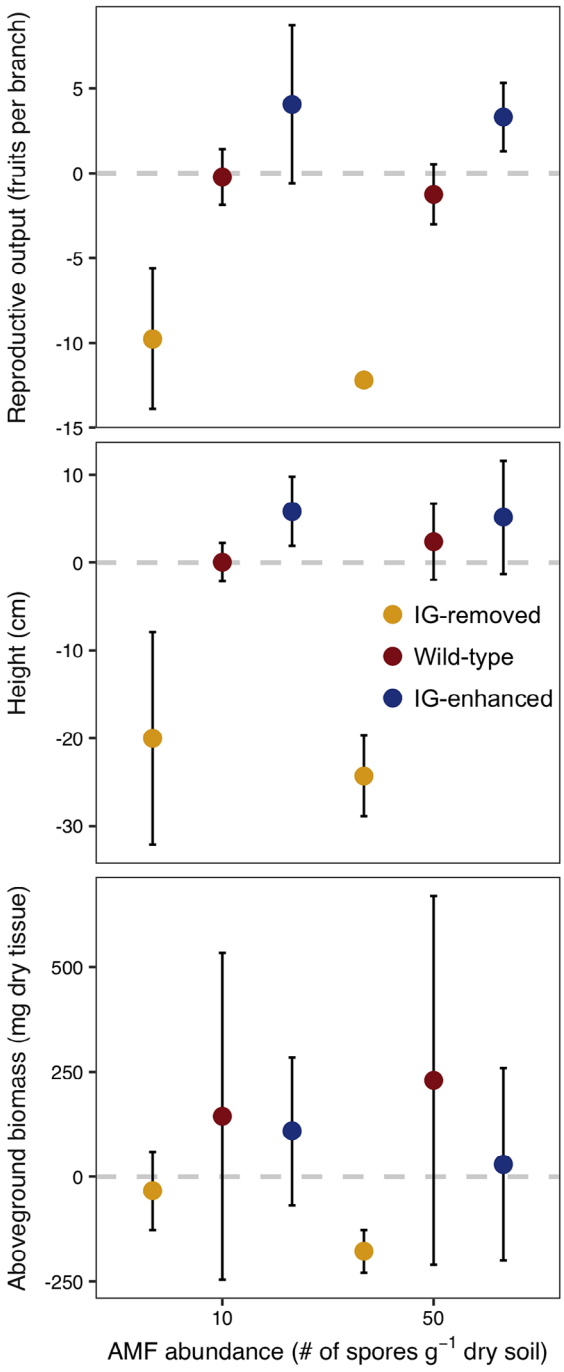

Fig. 3. Plant genotype performance across arbuscular mycorrhizal fungal inoculation levels. Values represent differences between inoculated and sterile controls. Points represent the mean difference and error bars are 95% confidence intervals. Confidence intervals that do not pass through zero are significantly different at the 5% level.

was significantly higher on IG-removed roots with AMF inoculation compared to wild-type or IG-enhanced plants (Fig. 4). Wild-type and IG-enhanced plants were never highly colonized by AMF regardless of growth environment (sterile vs. AMF inoculation).

Salicylic acid content was correlated to plant biomass for wild-type and IG-enhanced plants but not IG-removed plants (Table 1; Appendix S1: Fig. S1). Wild-type and IG-enhanced plant biomass were positively correlated to salicylic acid contents when grown with AMF, but not in the sterile soil treatment. Neither reproductive output nor plant height were correlated to salicylic acid content or vesicle colonization for wild-type or IG-enhanced plants. Plant

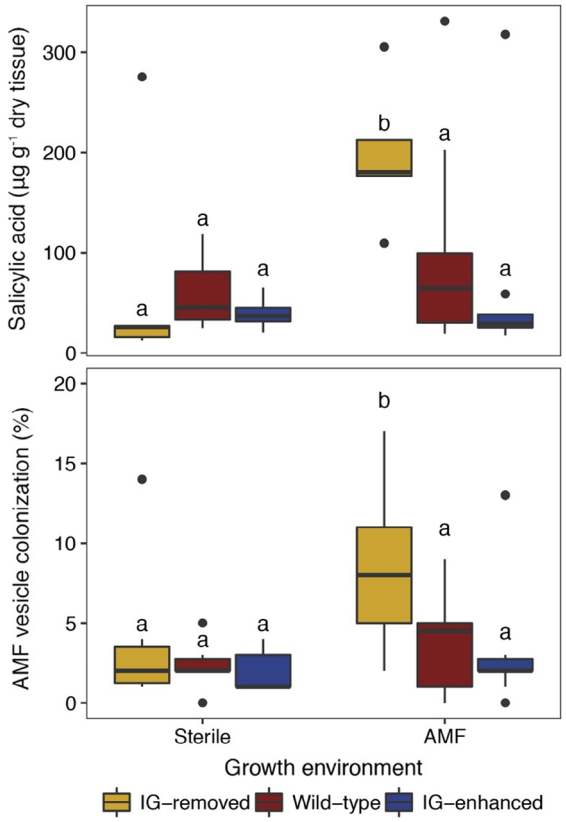

Fig. 4. Salicylic acid content and arbuscular mycorrhizal fungal (AMF) vesicle colonization in sterilized soil (no AMF) vs. soil with AMF inoculation. Bars represent the interquartile range, whiskers show the minimum and maximum values, points show suspected outliers, and horizontal black lines show the median. Bars with different letters are significantly different ($P \leq 0.05$) based on independent contrasts.

height was positively correlated to salicylic acid contents for IG-removed plants but not reproductive output. Vesicle colonization was not correlated to plant height nor reproductive output for any plants.

Discussion

There is strong evidence that the loss of conserved symbiosis related genes is responsible for non-functional arbuscular mycorrhizas in *Arabidopsis* (Delaux et al. 2014), but mechanisms by which *Arabidopsis* can defend itself against AMF-induced growth suppression are poorly understood. In this study, we show that the loss of two genes necessary for IG production in a mutant *Arabidopsis* line reduced resistance to AMF-induced growth suppression and was associated with higher AMF vesicle colonization compared to wild-type or mutant, IG-enhanced plants. These results support our general hypothesis that IGs are involved in suppressing AMF colonization on the root systems of *Arabidopsis*. We suggest that these results extend beyond the *Arabidopsis–Rhizophagus* system since AMF can colonize other species in the Brassicaceae (Hirrelet al. 1978), other AMF taxa besides *R. irregularis* can colonize non-host Brassicaceae (Regvaret al. 2003), and because IG orthologs are found across non-mycorrhizal species within the Brassicaceae.

IG-removed plant performance was reduced by AMF inoculation while wild-type and IG-enhanced plants were not affected

We hypothesized that AMF inoculation would suppress the growth, activate induced plant defenses, and promote AMF colonization on the root systems of IG-removed > wild-type > IG-enhanced plants. We found partial support for all three hypotheses. Notably, AMF inoculation reduced the performance, growth and reproductive output of the IG-removed plants while having no detrimental impact on wild-type or IG-enhanced plants. Since small concentrations of glucosinolates can suppress AMF germination (Cantor et al. 2011), the impact of AMF on *Arabidopsis* may not scale with IG production capacity within the range we detected.

We observed increased salicylic acid content in IG-removed plants exposed to AMF, which supports the prediction that AMF presence would activate plant defense systems in IG-removed plants. Indolic glucosinolate-removed plants may have over-invested in induced defenses, and this could be why their growth was suppressed by AMF inoculation since induced defenses are costly investments (Allen et al. 1989). Genotypes which produce IGs (i.e., wild-type and IG-enhanced) did not exhibit significantly elevated tissue salicylic acid contents. We hypothesize that this was a direct result of lower AMF root colonization on these genotypes since AMF vesicle colonization was higher on the root systems of IG-removed plants compared to wild-type and IG-enhanced plants. There was a

Table 1. Linear regressions between plant performance and salicylic acid contents and arbuscular mycorrhizal fungal (AMF) vesicle colonization in the AMF inoculation treatments.

Predictor variable	Reproductive output (no. fruits/branch)		Height (cm)		Biomass (mg dry tissue)	
	R^2	P	R^2	P	R^2	P
Salicylic acid(μg/g dry tissue)						
IG-removed	0.51	0.29	0.84	0.008 (+)	0.04	0.8
Wild-type	0.06	0.5	0.18	0.2	0.39	0.04 (+)
IG-enhanced	0.21	0.18	0.19	0.2	0.51	0.02 (+)
AMF vesicle colonization (%)						
IG-removed	0.05	0.77	0.11	0.58	0.31	0.33
Wild-type	0.07	0.42	0.13	0.27	<0.01	0.86
IG-enhanced	0.23	0.17	<0.01	0.85	0.29	0.11

Notes: Significant positive and negative correlations are indicated using + and −, respectively. Scatter plots are displayed in Appendix S1: Fig. S1. IG, indolic glucosinolates.

positive correlation between salicylic acid production and wild-type and IG-enhanced plant biomass in soils with AMF inoculation, but this was not observed for IG-removed plants. Plants with IG-producing capacities presumably invested balanced amounts in induced defenses, minimizing AMF colonization and increasing plant biomass. This observation suggests that IGs reduce AMF vesicle colonization on Brassicaceae roots, and in turn, protect plants from AMF-induced growth suppression via over-investments in induced defenses.

There is no reason to suspect that AMF colonization on *Arabidopsis* roots in our study provided typical mycorrhizal functions. Consistent with other work, root arbuscules were not observed on the roots of *Arabidopsis*, while vesicles were the most common root structure observed (Schreiner and Koide 1993, Regvaret al. 2003, Veigaet al. 2013). In other non-mycorrhizal plants, vesicles are commonly found on unhealthy or senescing roots and are unattached to internal hyphae (Glenn et al. 1985, Allen et al. 1989). It may be a sign of parasitism when vesicles, but no arbuscules are present to facilitate the allocation of carbon in Brassicaceae roots colonized by AMF. Tissue N contents also did not increase with AMF additions. Arbuscular mycorrhizal fungi can increase N-uptake in host plants (Hodge et al. 2001) and ammonium transporter genes are conserved among mycorrhizal host plants (Delaux et al. 2014), which indicates that there was not a functioning nutrient exchange in any of the AMF colonized plants. We did not measure tissue phosphorus contents, a nutrient which AMF typically supply to their host plants (Smith and Smith 2015), but the exchange of phosphorus for carbon would occur primarily in arbuscules (Manjarrez et al. 2010) which were not present. We cannot confirm that the vesicles were produced by *R. irregularis,* but because plants were grown in sterilized growth medium with a single species inoculant, this is the most probable explanation.

Limitations of the use of mutant lines

While our results indicate that IGs are involved in suppressing AMF colonization of Brassicaceae roots, we need to consider that the IG biosynthetic pathway also produces the auxin indole-3-acetic acid (IAA) and the defense compound camalexin. Thus, these mutant lines we used are pleiotropic. Indolic glucosinolate-removed plants produce 30% less of the IAA compared to the wild-type when grown at elevated temperatures (Zhao et al. 2002), and conversely, the IG-enhanced plants produce higher levels of IAA (Celenza et al. 2005). Nonetheless, these are relatively small changes in IAA which have minimal impacts on growth and development (Zhao et al. 2002, Celenza et al. 2005). Plus, IAA produced from the IG biosynthetic pathway is considered to be a minor contributor to the plant's IAA pool (Sugawara et al. 2009). Indole-3-acetic acid is also not known to be involved in AMF symbiosis (Danneberg et al. 1993, Ludwig-

Müller et al. 1997, Kaldorf and Ludwig-Müller 2000), although other auxins may increase AMF colonization on host plants roots (Fitze et al. 2005). If IAA is involved in facilitating AMF colonization, we would expect AMF colonization to be highest on the IG-enhanced plants which produce more IAA, which we did not find. The second modification to consider is that IG-removed plants also cannot produce camalexin while IG-enhanced plants produce more camalexin than the wild-type (Bender and Celenza 2009). Camalexin has anti-fungal properties (Nafisi et al. 2007), but it is not produced by plants unless elicited by a pathogen (Ferrari et al. 2003), unlike IGs that are constitutively present in most plant tissues (Zukalova and Vasak, 2002). Our findings are consistent with camalexin not having a role in AMF symbiosis because wild-type and IG-enhanced plants did not produce camalexin after inoculation with *R. irregularis*. When grown in sterile soil, IG-removed plants were also comparably fit and had the same tissue C and N and salicylic acid contents as wild-type and IG-enhanced plants, which suggests that the results we saw were not due to a sensitized background in the IG-removed plants. Nonetheless, we cannot isolate the direct impact of IGs based on our study design, and it is possible that other, unknown tryptophan-derived secondary metabolites from the IG pathway were involved.

There is a general loss of IG orthologs in mycorrhizal Brassicales which are present in non-mycorrhizal species

To assess whether these results may be applicable beyond *Arabidopsis*, we also determined whether AMF host plants and non-mycorrhizal species within the Brassicales produce similar proteins for IG production. This analysis is one way to assess whether mycorrhizal and non-mycorrhizal Brassicales possess distinct IG production capacities. We downloaded amino acid sequences which encode for IG-related proteins (see Celenza et al. 2005 and Bender and Celenza 2009) using the gene names cyp79b2, cyp79b3, cyp83b1, MYB121, MYB34, and MYB51 from the TAIR database (Appendix S1: Table S3; Berardini et al. 2015). We then conducted BLASTp and tBLASTn searches against 29 species of Brassicales of previous interest (Delaux et al. 2014, Cosme et al. 2014, or Vierheilig et al. 2000) or species with known mycorrhizal statuses, as determined using published literature, and complete genomes in GenBank or PhytoZome.

We found that plants characterized as non-mycorrhizal are the only species which possess all six IG-related orthologs (Fig. 5), and thirteen of 14 non-mycorrhizal species contain necessary CYP-related proteins for IG production. Non-mycorrhizal, *Crambehispanica* lacks IG orthologs. IGs are also not present in the tissues of *C. hispanica* (Warwick and Gugel 2003). Why this species stands out is not obvious, but whether *C. hispanica* is truly non-mycorrhizal (DeMars and Boerner 1996) should be re-examined since these designations can be inconsistent in the literature (Cosme et al. 2018). Conversely, 12 of the 15 mycorrhizal plants completely lacked IG orthologs. The mycorrhizal *Carica papya* is able to produce IGs *albeit* lacking a MYB51 ortholog. This may explain why IGs are not always present in *C. papaya* (Vierheilig et al. 2000) or at low levels (Ludwig-Müller et al. 2002). Mycorrhizal *Sisymbrium irio* and *Euclidium syriacum* also possessed CYP proteins but not MYB orthologs. Indolic glucosinolates are not reported in *S. irio* (Fahey et al. 2001), though IGs orders of magnitude lower than reported for *Arabidopsis* in this study have been detected following powdery mildew exposure (Bednarek et al., 2011). Conversely, *E. syriacum* is not reported to produce IGs (Brown and Morra 2005). Our results suggest that a lack of MYB transcription factors in mycorrhizal Brassicales may minimize or inhibit IG production and remove barriers for AMF colonization in these mycorrhizal plants.

These findings support our experimental work using *Arabidopsis* and suggest that IGs differentiate mycorrhizal and non-mycorrhizal Brassicales. However, incomplete genomic data for some of the species make it impossible to draw firm conclusions as to whether species truly lack IG orthologs, but this would apply to both mycorrhizal and non-mycorrhizal species analyzed. For example, *T. majus* can produce small amounts of IGs (Ludwig-Müller et al. 2002), but no IG orthologs were found for this species. This was the only species for which transcriptome data were available, and this can explain this result since *T. majus* does not produce IGs constitutively (Ludwig-Müller et al. 2002) and IG transcripts would not be synthesized unless induced by a pathogen, herbivore, or potentially AMF. It

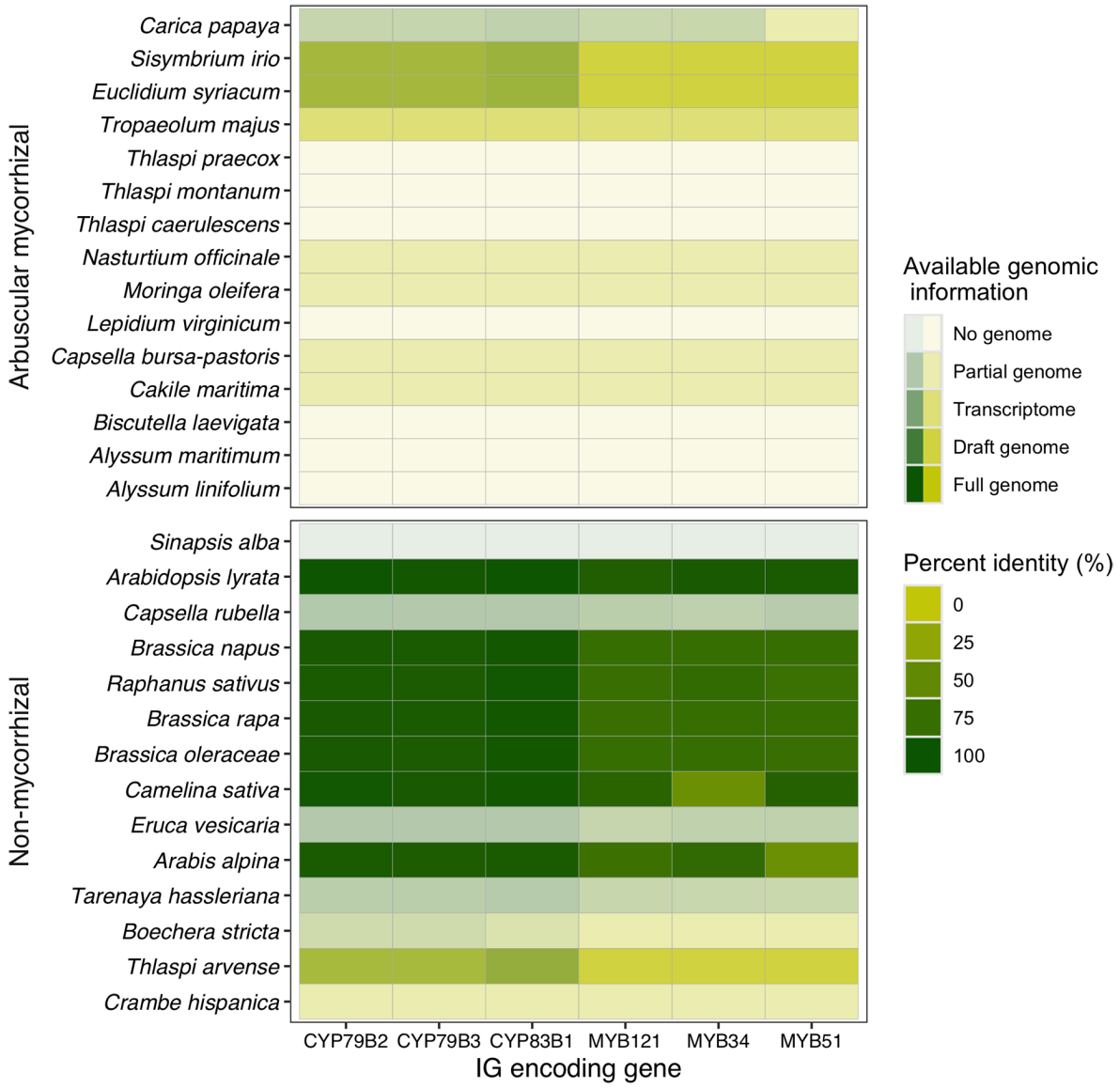

Fig. 5. Orthologous indolic glucosinolate proteins between *Arabidopsis thaliana* and 29 members of the Brassicales. The availability of genomic information in NCBI and JGI for each species is shown using transparency. Genomic information included species with no known genome project but which had protein sequence data in the databases (no genome), a started and incomplete genome (partial), transcriptome data (transcriptome), a complete, published draft genome (draft), and complete genomes (full). Percent identity between the *Arabidopsis* amino acid sequence and the lowest E-value hit based on BLASTp or tBLASTn searches is shown. The mycorrhizal status of each plant species is based on published observations. Complete information on the BLAST results, genomic information, and mycorrhizal host plant status of each species is detailed in Appendix S1: Table S4.

is notable that non-mycorrhizal plants with incomplete genomic information (e.g., *Sinapsis alba, Capsella rubella, Eruca vesicaria, Boechera strict*) possess IG orthologs while mycorrhizal plants with comparably incomplete information do not. The distribution of IG proteins, and the completeness of these proteins, therefore differentiates mycorrhizal and non-mycorrhizal

Brassicales and supports our experimental results that IGs are involved in suppressing AMF colonization on non-host Brassicaceae.

Conclusion

In conclusion, our results provide evidence that the IG pathway facilitates resistance to AMF colonization in *Arabidopsis*. This is consistent with the IG pathway being involved in suppressing fungal pathogens and endophytes (Bednarek et al. 2009; 2011; Hiruma et al. 2010; Schlaeppi et al. 2010; Nongbri et al. 2012; Zhang et al. 2015). Future work will need to assess whether the results of our study apply to other Brassicaceae but we suspect so since IG-producing orthologs are found across different phylogenetic branches of the Brassicaceae. Our results therefore have potential agricultural applications. If Brassicaceae are grown in soils where AMF abundances are sufficiently high then the IG production capacity of the plant should be considered. Certain commonly grown canola (*Brassica napus*) cultivars produce 10× the IG levels of low IG cultivars (Shahidi and Gabon, 1989). A major goal of oil-seed Brassicaceae breeding has been to reduce glucosinolate levels because they can decrease palatability and cause thyroid and liver damage, the latter being of particular concern for oil-seed crops (e.g., canola) whose byproducts are used in animal feed (Fahey et al. 2001, Anilakumar et al. 2006). However, for Brassicaceae vegetables, increasing IG levels has been a domestication goal because of beneficial anticarcinogenic effects (Prakah and Gupta 2012). Depending on the crop (oil-seed vs. vegetable), optimum plant breeding should be considered in relation to AMF levels in soil in order to optimize plant growth and the positive vs. negative nutritional benefits of IGs.

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Supporting Information

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