

# *Metarhizium robertsii* Produces an Extracellular Invertase (MrINV) That Plays a Pivotal Role in Rhizospheric Interactions and Root Colonization

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## Abstract

As well as killing pest insects, the rhizosphere competent insect-pathogenic fungus *Metarhizium robertsii* also boosts plant growth by providing nitrogenous nutrients and increasing resistance to plant pathogens. Plant roots secrete abundant nutrients but little is known about their utilization by *Metarhizium* spp. and the mechanistic basis of *Metarhizium*-plant associations. We report here that *M. robertsii* produces an extracellular invertase (*Mrlnv*) on plant roots. Deletion of *Mrlnv* ( $\Delta Mrlnv$ ) reduced *M. robertsii* growth on sucrose and rhizospheric exudates but increased colonization of *Panicum virgatum* and *Arabidopsis thaliana* roots. This could be accounted for by a reduction in carbon catabolite repression in  $\Delta Mrlnv$  increasing production of plant cell wall-degrading depolymerases. A non-rhizosphere competent scarab beetle specialist *Metarhizium majus* lacks invertase which suggests that rhizospheric competence may be related to the sugar metabolism of different *Metarhizium* species.

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## Introduction

The entomopathogenic fungus *Metarhizium robertsii* (formerly known as *Metarhizium anisopliae* var *anisopliae* [1]) is currently applied as a biological control agent against various insect pests [2-4]. Recent investigations have revealed that species of *Metarhizium* which are widely distributed in soil also play other ecological roles, including establishing mutualistic interactions with plants as rhizospheric associates [5]. Seed treatment with these *Metarhizium* spp. increases crop yields by killing soil insects [6], inhibiting plant pathogens [7], enhancing the uptake of micronutrients [8], and translocating nitrogen from killed insects to plants [9]. However, *Metarhizium* has yet to be exploited in agriculture as a plant growth enhancer and the mechanistic basis for its many interactions are poorly understood. The mycoparasite *Trichoderma* is the best studied plant root colonizer and the most widely applied fungal plant growth promoter [10,11]. Much effort and expense has been directed at finding better ways to apply both *Metarhizium* spp. (as insecticides) and *Trichoderma* spp. (as plant symbionts). However, as with *Metarhizium*, exploitation of *Trichoderma* has been handicapped by inconsistent field performance [12,13].

More consistent biopesticide products might be achieved based on combinations of microbes that economically achieve breadth of action. To this end, identifying commonalities and differences between *Metarhizium* and *Trichoderma* in their mode of action could be used to facilitate synergistic effects when applied together as a seed treatment. Comparative genomics reveals *Metarhizium* and *Trichoderma* are related [14], but studies to date have shown mechanistic differences in root colonization that suggest they have independently evolved rhizosphere competence. For instance, *Trichoderma* uses a hydrophobin to adhere to roots [15], whereas *Metarhizium* uses an adhesin [16].

Nevertheless, given their shared habitat there should also be similarities and some of these could potentially lead to competition for resources. In soils, both *Metarhizium* and *Trichoderma* exploit various products of plant photosynthesis. The ability to respond to and utilize these products will likely determine their success as plant root colonizers. Sucrose is one of the main carbohydrate products of photosynthesis and has been detected in high concentrations near root tips [17-19]. Several fungi such as *Thermomyces lanuginosus*, *Uromyces fabae*, *Aspergillus niger* and *Trichoderma virens* produce

invertases capable of hydrolyzing sucrose to monosaccharides [20–23]. In *U. fabae*, invertase is highly expressed in haustoria during the process of infection in the leaf. Furthermore, plant-derived sucrose is a key component in symbiotic associations between *T. virens* and maize [22].

In the present study, a gene (*Mrlnv*) with homology to the intracellular invertase of *T. virens* was identified and characterized in *M. robertsii*. *Mrlnv* encodes a single copy extracellular invertase that is principally responsible for sucrose hydrolysis by *M. robertsii*. Disrupting the gene resulted in poor fungal growth on sucrose, root exudates and rhizospheric soils. However, root colonization by the mutant was increased. We present evidence that this resulted from increased expression of cell wall-degrading enzymes because of reduced carbon catabolite repression in the mutant. Our data suggest that while *Mrlnv* plays an important role in providing the fungus with a carbon source, this limits the extent of root colonization during *Metarhizium*-plant associations.

## Materials and Methods

### Living Materials and Culture Conditions

*Metarhizium robertsii* ARSEF 2575 and *M. majus* ARSEF 297 wild-type strains (USDA/ARS Collection) were grown and maintained on Potato Dextrose Agar (PDA) (Fluka, USA) at 27 °C. *Escherichia coli* DH5 $\alpha$  and *Agrobacterium tumefaciens* AGL-1 were used for DNA cloning and fungal transformation. *Arabidopsis thaliana* eco-type Col-0 seeds were purchased from LEHLE SEEDS (Round Rock, Texas, USA). *Panicum virgatum* (switchgrass) seeds were obtained from OSC seeds (Waterloo, Ontario, Canada). *A. thaliana* and switchgrass seeds were surface-disinfected according to Sauer and Burroughs [24] and Miché and Balandreau [25], respectively. The sterilized seeds were kept at 4 °C overnight to allow for synchronization of growth before fungal inoculation.

### Gene Disruption

A *Mrlnv* disruption vector pPK2BargfpDMrlnv was constructed to knock out the *Mrlnv* gene in *M. robertsii*. The 5'-end and 3'-end of *Mrlnv*, cloned by PCR, were inserted into a modified master Ti vector pFBarGFP using the *Xba* I and *Bgl* III/*Eco*R V sites, respectively. The disruption mutant ( $\Delta$ *Mrlnv*) was obtained by *A. tumefaciens*-mediated transformation [26]. To complement  $\Delta$ *Mrlnv*, a ~ 3.9 kb genomic fragment containing the *Mrlnv* open reading frame and its flanking sequence was cloned and inserted into the *Eco*R V site of pPK2SurGFP with the *Sur* selective marker under control of a constitutive glyceraldehyde-3-phosphate dehydrogenase (*Pgpd*) promoter [27,28]. The  $\Delta$ *Mrlnv* revertant ( $\Delta$ *Mrlnv*-rv) was obtained by transforming the construct into  $\Delta$ *Mrlnv*.

### qPCR Analysis

To quantify gene expression in response to sugars, wild-type *M. robertsii* and *M. robertsii*  $\Delta$ *Mrlnv* were grown in Sabouraud Dextrose Broth (SDB, Difco) for 30 h, and mycelia were transferred to minimal medium (M100 medium minus glucose)

plus 1% sucrose (sucrose cultures). To observe the time course of *Mrlnv* expression, RNA was harvested from mycelia for qPCR analysis as described previously [29]. The primers used in this study are listed in Table S1.

### Sugar Utilization Assay

The ability of  $\Delta$ *Mrlnv* and wild-type strains to utilize different sugars was analyzed according to a modification of the method of Fang and St. Leger [30]. Sterile distilled water plus or minus a nitrogen source (0.1% NaNO<sub>3</sub>) was combined with a carbohydrate (0.1%). The soybean root exudate was prepared as described by Pava-Ripoll et al. [31]. To determine growth rates, mycelial inoculums (0.2 g wet mycelia, approximately 25 mg dry biomass) from SDB cultures were transferred into liquid minimal medium supplemented with 1% sucrose and/or 1% glucose. Mycelia harvested after 12 h growth was used for dry weight determinations.

### Rhizosphere Competence and Root Colonization Assays

Sterile synchronized *P. virgatum* (switchgrass) seeds were inoculated by immersion for 1 h in  $1 \times 10^9$ /mL<sup>-1</sup> *M. robertsii* wild-type or  $\Delta$ *Mrlnv* spores as described by Wyrebek et al. [32]. The seeds were planted in pots (0.5 g seeds per pot) filled with sterile soil (Scotts Turf Builder Seeding Soil, Scotts Company, USA). Each treatment was replicated three times. To determine fungal survival in bulk soil with or without switchgrass seeds, spore suspensions were spread evenly through soil producing approximately  $5 \times 10^3$  spores/g<sup>-1</sup> soil. Half the pots were planted with uninoculated seeds. All the pots were kept in a growth chamber at 25 °C with 14:10 h light:dark cycle, and within two weeks the grass had produced a lawn covering the soil surface. Sterile water was added regularly to avoid drying. The soil population of wild-type and  $\Delta$ *Mrlnv* strains was monitored at set intervals by a slight modification of Fang and St. Leger [30]. Briefly, 0.5 g of soil from each pot was collected using a cork borer. This soil contained a high density of roots so the fungus would be existing in overlapping rhizospheres. Soil suspensions were prepared by adding 5 mL 0.05% Tween 80 solution and vortexing vigorously. Aliquots (100  $\mu$ L) were spread on Rose-bengal selective agar plates and CFUs were determined after 10 days at 27 °C [5].

To assay root colonization, 10-d-old *A. thaliana* seedlings were inoculated in liquid Murashige and Skoog medium (Sigma, USA) containing  $5 \times 10^6$  spores mL<sup>-1</sup> *M. robertsii* suspensions. After 48 h incubation, *A. thaliana* roots were collected, and washed three times in 0.05% Tween 80 solution. To observe fungal colonization, individual *A. thaliana* root were stained with fungi lactophenol cotton blue (ENG Scientific, Inc., USA) and mounted on a slide for microscopy. *M. robertsii* root colonization was quantified by a modification of Viterbo et al. [33]. To quantify wild-type and mutant growth, three replicates each containing nine *A. thaliana* roots were weighed and homogenized by vortexing vigorously for 2 min in 500  $\mu$ L 0.05% Tween 80 solution. Serial dilutions were assayed for CFU on Rose-bengal selective agar plates [5]. The same procedure was applied on switchgrass except that each replicate contained three switchgrass roots from each pot.

Roots were weighed and ground with mortar and pestle in 2 mL 0.05% Tween 80 solution. The CFUs were quantified as described above.

### Enzymatic Assays

Enzymatic activities were assayed in filtrates from cultures grown with sucrose or *A. thaliana* seedlings as described previously. The Pr1 subtilisin protease activity was assayed with N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide [34]. One unit of protease activity was defined as the amount of enzyme that produces 1  $\mu$ M of para-nitro aniline per minute. Invertase and endochitinase activities were assayed using the EnzyChrom™ Invertase Assay Kit (BioAssay Systems, USA) and the Chitinase Assay Kit, Fluorimetric (Sigma, USA). Total protein content was measured with a Protein Assay Kit II (Bio-Rad, USA). Pectinase activity versus polygalacturonic acid was assayed according to the protocol from Worthington Biochemical Corporation (New Jersey, USA). One unit of pectinase was defined as the amount of enzyme that liberates 1  $\mu$ mole of D-galacturonic acid from polygalacturonic acid per minute.

### Plant Growth

To assess the effect of *M. robertsii* on switchgrass, stem lengths were measured and leaf chlorophyll content was read using a SPAD-502 Plus chlorophyll meter (Konica Minolta Sensing, Inc. Japan). The plants were harvested after three months, and rhizospheric soils washed off the roots, and root lengths measured as described previously [5]. The dry weight of whole plants was determined as a measure of biomass.

## Results

### Identification and Characterization of an Invertase MrINV in *M. robertsii*

Using an invertase TvINV from *T. virens* (accession no. EHK21605) as query, we identified a single homolog (*Mrlnv*) in the *M. robertsii* genome [14] with a maximum identity of 41% ( $5e^{-7}$ ). The Open Reading Frame (ORF) of *Mrlnv* is 1782-bp long coding for a putative 573 amino acid protein with a predicted molecular weight of 63.9 kDa, and is interrupted by one 60-bp intron. As deduced by SignalP 4.1 [35], MrINV contains a predicted 24-aa signal peptide for secretion. The predicted cleavage site is between G24 and H25 and the calculated molecular weight of the mature protein is 61.3 kDa. The signal peptide resembled those on extracellular invertases from bacteria and some fungi including yeasts. Phylogenetic reconstruction grouped fungal invertases into two separate clades (Figure 1). MrINV grouped with fungal invertases containing a NDPN box (conserved  $\beta$ -fructosidase motif) [36]. However, no homologous sequence was found in the scarab beetle specialist *M. majus*.

To better understand the role of *Mrlnv* during *M. robertsii* development, regulation of *Mrlnv* was determined under different cultural conditions using quantitative reverse transcription PCR (qPCR). Total RNA was isolated from mycelia grown on different carbohydrates including

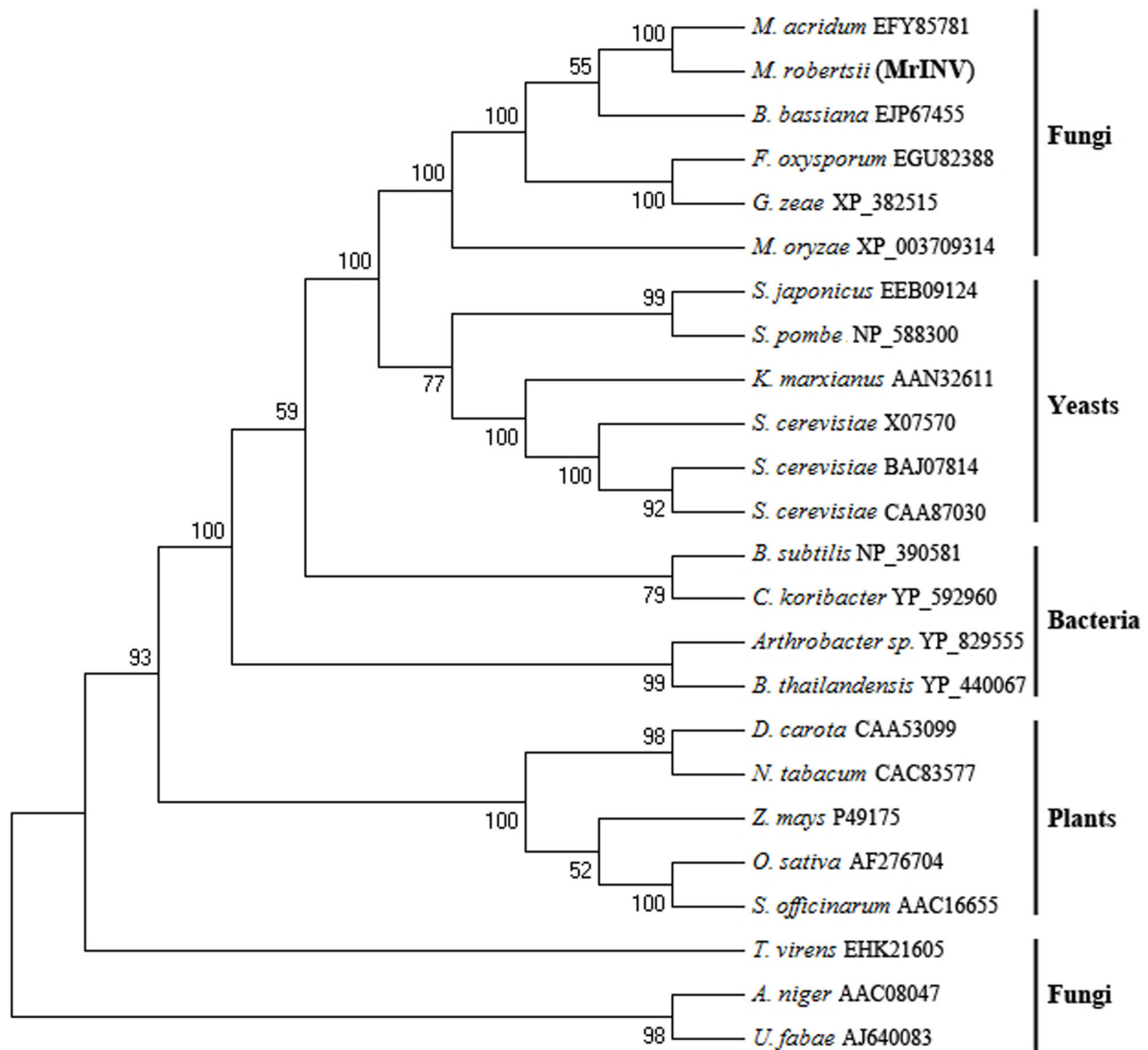
monosaccharides, homologous disaccharides and heterologous dioligosaccharides (Table 1). *Mrlnv* expression was up-regulated 40-fold in the presence of sucrose (Figure 2A), but other carbohydrates had no effect on expression (data not shown). A time-course for *Mrlnv* expression in sucrose culture showed onset at 20 minutes, and a peak at 4 h which then plateaued (Figure 2B).

### *Mrlnv* Disruption Suppressed *M. robertsii* Growth on Sucrose

To confirm that MrINV is a functional invertase involved in sucrose metabolism in *M. robertsii*, we produced a null mutant ( $\Delta$ *Mrlnv*), deletion of the wild-type allele. To complement  $\Delta$ *Mrlnv*, the genomic fragment of *Mrlnv* was transformed into  $\Delta$ *Mrlnv* resulting in  $\Delta$ *Mrlnv-rv*. The deletion of *Mrlnv* in  $\Delta$ *Mrlnv* was confirmed by PCR using the genomic DNA as template (Figure S1). RT-PCR confirmed there was no *Mrlnv* expression in  $\Delta$ *Mrlnv*, and as expected,  $\Delta$ *Mrlnv* showed no invertase activity during 8 h growth on sucrose. Invertase activity was detected in the wild-type strain and  $\Delta$ *Mrlnv* revertant ( $\Delta$ *Mrlnv-rv*) (Figure 2C), confirming that *Mrlnv* encodes the invertase up-regulated in the presence of sucrose.

To determine the role of *Mrlnv*, the wild-type and  $\Delta$ *Mrlnv* strains were compared in colony morphology, germination rate and growth. Conidial germination of  $\Delta$ *Mrlnv* ( $8.0\% \pm 1.2\%$ ) after 24 h in sucrose medium was reduced by 61% compared to the wild-type strain ( $20.7\% \pm 1.2\%$ ) (Table 1,  $P < 0.01$ ). Likewise, colony growth (Figure S2) and biomass in sucrose medium were significantly reduced in  $\Delta$ *Mrlnv* compared to the wild-type strain (Figure 3,  $P < 0.01$ ). The addition of an inorganic nitrogen source ( $\text{NaNO}_3$ ) did not affect the ability of *Mrlnv* to utilize sucrose, and  $\Delta$ *Mrlnv* and the wild-type strain germinated at similar rates in other carbohydrates, including glucose (Table 1). In addition,  $\Delta$ *Mrlnv* grew at a similar rate as the wild-type strain using glucose as sole carbon source (Figure S3). Thus, the only impairment of  $\Delta$ *Mrlnv* is in its ability to utilize sucrose which confirms MrINV is a functional invertase.

To determine whether MrINV is critical for sucrose utilization, the germination rate of  $\Delta$ *Mrlnv* and *M. robertsii* wild-type on sucrose was compared with *M. majus* (lacks an invertase homolog). After 24 h incubation, *M. majus* showed a significantly lower germination rate ( $2.3\% \pm 0.3\%$ ) on sucrose compared with  $\Delta$ *Mrlnv* ( $7.7\% \pm 0.3\%$ ) and *M. robertsii* wild-type ( $16.0\% \pm 1.0\%$ ) (Figure 4,  $P < 0.01$ ). Combining sucrose with a nitrogen source ( $\text{NaNO}_3$ ), elevated *M. majus* germination ( $7.0\% \pm 1.2\%$ ) to a similar level as  $\Delta$ *Mrlnv* ( $9.0\% \pm 0.6\%$ ) but still lower than *M. robertsii* wild-type ( $17.0\% \pm 2.5\%$ ) (Figure 4,  $P < 0.05$ ). Though lacking invertase,  $\Delta$ *Mrlnv* and *M. majus* strains can still grow poorly on sucrose as sole carbon source which suggests that *Metarhizium* spp. has additional less efficient mechanisms for metabolizing sucrose for growth and development. This contrasts with *T. virens* as invertase is its sole means of utilizing sucrose [22].



**Figure 1. Neighbour-joining (NJ) tree of invertase from *M. robertsii* (MrINV) and other 23 invertases from bacteria, plants, yeasts and other fungi.** Amino acid sequences were aligned using the ClustalX algorithm and the NJ tree was constructed with the MEGA4 software. Bootstrap values are adjacent to each internal node, representing the percentage of 1,000 bootstrap replicates.

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### ***MrInV* Disruption Impaired *M. robertsii* Rhizosphere Competency but Enhanced Root Colonization**

To analyze the involvement of *MrInV* in the rhizosphere competency of *M. robertsii*, we investigated fungal survivorship in the soil and measured root colonization. Firstly, to mimic rhizospheric nutrient sources we used soybean root exudate, which contains plant-derived sucrose as a major component [31]. No difference in the germination rate was observed between  $\Delta MrInV$  and wild-type strains at high concentration of root exudate (1 mg mL<sup>-1</sup>). But at low concentrations (0.1 mg

mL<sup>-1</sup> and 0.01 mg mL<sup>-1</sup>), germination of  $\Delta MrInV$  was reduced by 16% and 56%, respectively (Table 1,  $P < 0.01$ ).

The poor growth of  $\Delta MrInV$  in root exudate implied that  $\Delta MrInV$  may be impaired in rhizosphere competency. To investigate rhizospheric interactions and fungal root colonization, spores of  $\Delta MrInV$  and wild-type strains were inoculated into soil microcosms containing switchgrass. Switchgrass is easy to culture in lab conditions and is a well characterized host for *M. robertsii* [32]. The rhizosphere competency of  $\Delta MrInV$  and wild-type strains was determined by counting colony-forming units (CFUs) in soil samples [30].

**Table 1.** *In vitro* growth in different carbohydrates and root exudates.

	wt	$\Delta Mrlnv$	$\Delta Mrlnv-rv$
dH <sub>2</sub> O	1.3 ± 0.3	1.7 ± 0.3	1.5 ± 0.6
Sucrose	20.7 ± 1.2 <sup>A</sup>	8.0 ± 1.2 <sup>B</sup>	19.5 ± 0.9 <sup>A</sup>
Raffinose	6.3 ± 0.9	6.7 ± 1.8	7.0 ± 0.5
Fructose	44.0 ± 2.0	45.0 ± 2.3	42.9 ± 1.1
Glucose	47.1 ± 1.1	45.8 ± 1.8	46.5 ± 1.3
Maltose	6.7 ± 1.9	9.0 ± 1.7	8.1 ± 1.2
NaNO <sub>3</sub>	8.7 ± 0.3	9.7 ± 0.9	9.2 ± 1.0
NaNO <sub>3</sub> + Sucrose	19.7 ± 1.9 <sup>A</sup>	10.0 ± 0.6 <sup>B</sup>	19.1 ± 1.4 <sup>A</sup>
NaNO <sub>3</sub> + Raffinose	12.0 ± 0.6	12.0 ± 1.5	11.3 ± 0.9
NaNO <sub>3</sub> + Fructose	47.3 ± 3.8	46.3 ± 5.6	45.9 ± 2.3
NaNO <sub>3</sub> + Glucose	46.8 ± 0.9	46.5 ± 2.1	47.9 ± 1.7
NaNO <sub>3</sub> + Maltose	13.3 ± 0.3	14.7 ± 1.3	15.0 ± 1.3
<b>Root exudates</b>			
1 mg mL <sup>-1</sup>	98.0 ± 0.6	98.3 ± 0.7	99.0 ± 1.0
0.1 mg mL <sup>-1</sup>	79.0 ± 0.6 <sup>A</sup>	66.3 ± 2.7 <sup>B</sup>	80.9 ± 1.8 <sup>A</sup>
0.01 mg mL <sup>-1</sup>	51.3 ± 2.3 <sup>A</sup>	22.3 ± 1.2 <sup>B</sup>	50.1 ± 1.5 <sup>A</sup>

Upper-case letters represent means statistically different at the 0.01 level.

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Initial densities were determined by counting CFUs immediately after adding fungal spores. Two weeks post-inoculation the number of  $\Delta Mrlnv$  and wild-type CFUs had dropped by half. Two months post-inoculation,  $\Delta Mrlnv$  levels were still 37% less than the initial density, but the number of wild-type CFUs had increased 2.4-fold. At three months,  $\Delta Mrlnv$  and wild-type rhizospheric populations were 2.5-fold and 8-fold higher, respectively. Thus, compared to the wild-type, the number of  $\Delta Mrlnv$  CFUs were 72%, 75% and 71% less in the first, second and third month post-inoculation, respectively (Figure 5,  $P < 0.01$ ). CFU counts of  $\Delta Mrlnv$  and wild-type strains in bulk soil (soil containing fungi but no seeds) declined at the same rate over 3 months. The significant reduction of  $\Delta Mrlnv$  in the rhizosphere microcosms relative to the wild-type suggests *Mrlnv* is important for rhizosphere competency through utilization of plant-derived sucrose as carbon source.

As well as colonizing the rhizosphere, *M. robertsii* also grows on root surfaces [30]. Since deletion of *Mrlnv* reduced rhizosphere competency, we also determined if *Mrlnv* facilitates colonization of roots by counting CFUs extracted from switchgrass roots. Deleting *Mrlnv* produced a 2.7-fold increase in root colonization relative to the wild-type strain (Figure 6A,  $P < 0.05$ ). To determine if the increased root colonization by  $\Delta Mrlnv$  on switchgrass is applicable to other plants, *A. thaliana* roots were inoculated with  $\Delta Mrlnv$  or wild-type strain in a hydroponic system. Root colonization was determined after 48 h incubation by counting CFUs. Colonization of *A. thaliana* roots by  $\Delta Mrlnv$  was increased 2.1-fold relative to the wild-type strain (Figure 6B,  $P < 0.01$ ). Microscopic observation confirmed that  $\Delta Mrlnv$  hyphae proliferated more than wild-type hyphae on *A. thaliana* roots (Figure 6C).

### *Mrlnv* Disruption reduces Carbon Catabolite Repression and Increases Hydrolytic Enzyme Activity

During *Metarhizium*-plant associations, hyphae penetrate the superficial cell layers [30]. To breach the epidermic cell wall, fungi secrete hydrolytic enzymes such as pectinase [37]. Likewise, entomopathogenic fungi produce cuticle degrading proteinases and endochitinases that target the integuments of their insect hosts [38–40]. We compared production of hydrolytic enzymes by the  $\Delta Mrlnv$  and wild-type strains when colonizing roots. Higher levels of pectinase (Figure 7A,  $P < 0.05$ ), Pr1 (Figure 7B,  $P < 0.05$ ) and endochitinase (Figure 7C,  $P < 0.01$ ) activities were produced by  $\Delta Mrlnv$  in the hydroponic system containing *A. thaliana* seedlings.

Production of many hydrolytic enzymes in fungi is regulated by carbon catabolite repression (CCR) [41]. We hypothesized that hydrolytic enzymes are up-regulated in  $\Delta Mrlnv$  due to less glucose uptake and thereby reduced CCR. To test this we measured the expression of CCR-related genes. Consistent with greater CCR, sucrose induced higher levels of the CCR-related genes *tps1* (8.3-fold) and *hvk1* (2.6-fold) in the wild-type than in  $\Delta Mrlnv$  (Figure 8,  $P < 0.01$ ). The current study suggests that hydrolytic enzymes from *M. robertsii* are involved in the root colonization process and the hydrolytic enzyme activities in *Mrlnv* deficiency strain are up-regulated possibly through the release of CCR.

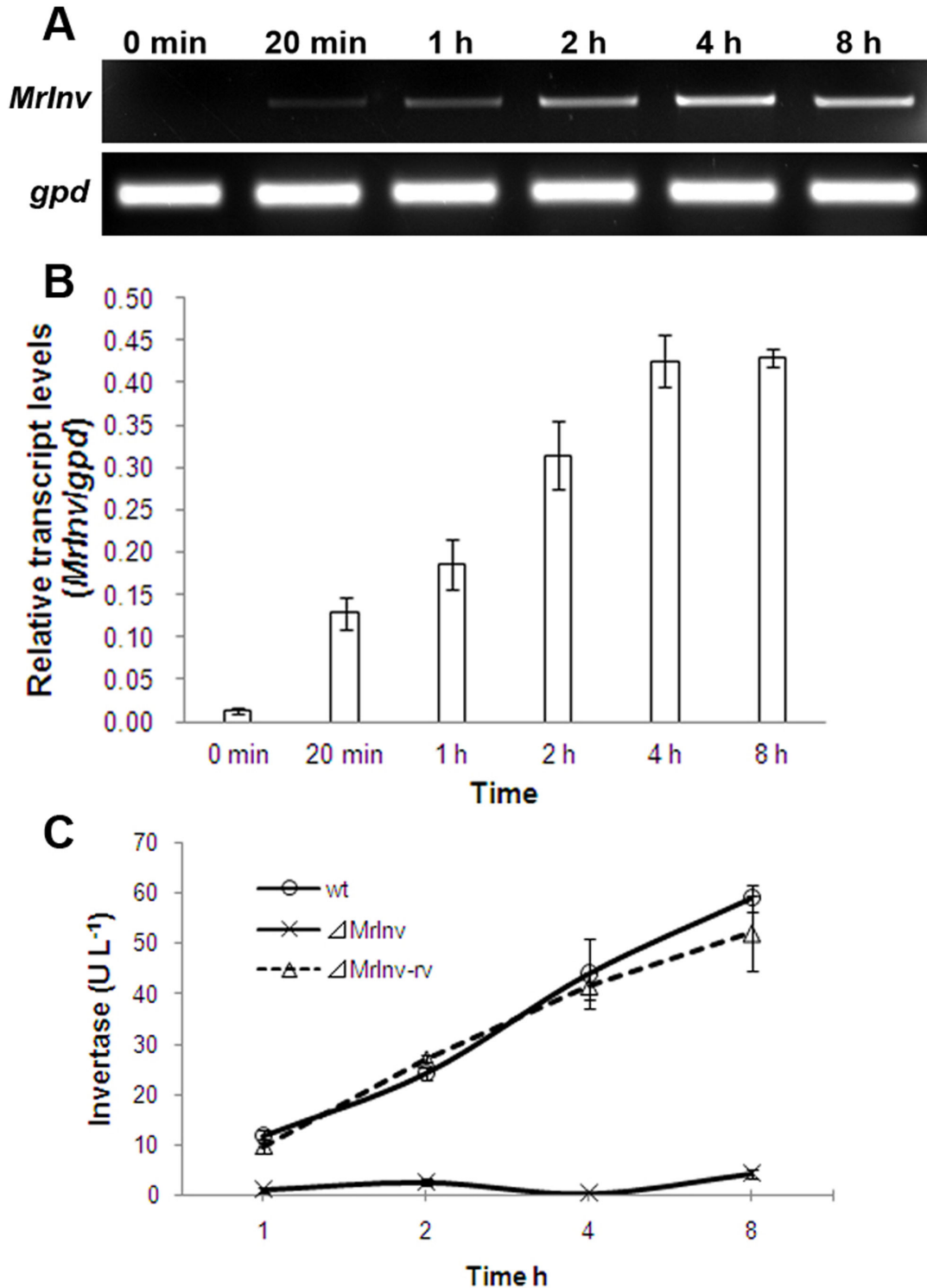
### Plant growth

The growth of switchgrass inoculated with  $\Delta Mrlnv$  and wild-type strains was monitored for 3 months at one month intervals to determine whether *Mrlnv* expression had an impact on plant growth. No differences were found between plants inoculated with  $\Delta Mrlnv$  and wild-type strain in stem length, leaf chlorophyll content, root length and plant biomass (Figure S4). In addition, the growth of switchgrass uninoculated with either fungi was similar with the plants inoculated with *M. robertsii*.

### Discussion

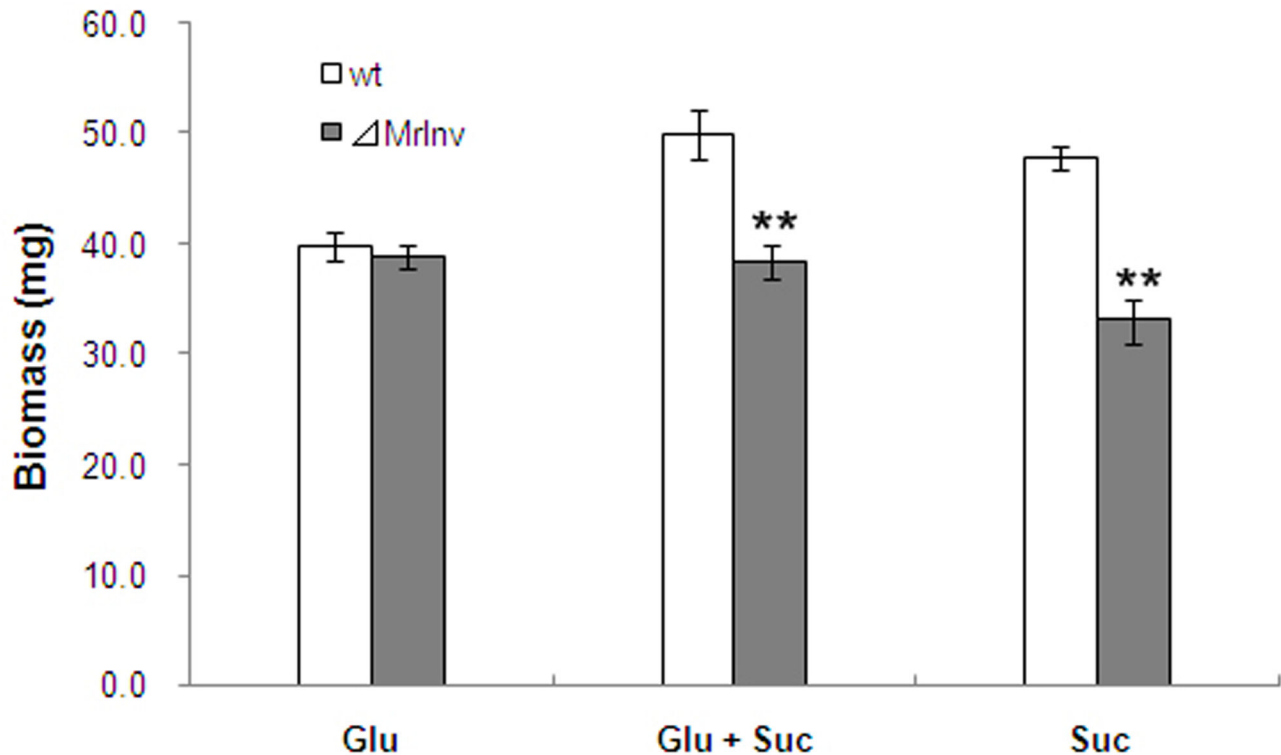
Enormous numbers of microbes inhabit the rhizosphere using various products of plant photosynthesis, and in return some microbes boost plant growth and health through several mechanisms including phyto-stimulation, biofertilisation, bioremediation and biological control [42]. The rhizosphere competence of *M. robertsii* was discovered comparatively recently in a field plot of cabbages [5]. *M. robertsii* is the only *Metarhizium* species found to associate with grass roots in the field [32], but in this study, we found that both switchgrass and the dicotyledonous *A. thaliana* supported extensive root colonization by *M. robertsii*.

Compared to interactions between fungal pathogens and their hosts, little is known about the molecular mechanisms underlying the complex interactions between roots, root exudate and any rhizospheric fungus, including the best studied examples in the genus *Trichoderma*. It is particularly important to comprehensively understand the natural ecological role of *Metarhizium* and *Trichoderma* in order to optimize their use as biological control agents. Table 2 summarizes currently understood similarities and differences between *Metarhizium*



**Figure 2. Characteristics of *MrInv* in *M. robertsii*.** A, Expression assay with qPCR after mycelia transferred from SDB to minimal medium plus 1% sucrose, grown at 27 °C for 8 h. B, Relative transcript levels of *MrInv* gene versus the housekeeping *gpd* gene. *gpd*, glyceraldehyde-3-phosphate dehydrogenase gene. Values are means of three replicates with corresponding standard deviation. C, The invertase activity of *M. robertsii* 2575 wild-type strain,  $\Delta$ *MrInv* and  $\Delta$ *MrInv* revertant. Mycelia were transferred from SDB to minimal medium plus 1% sucrose, grown at 27 °C for 8 h. The filtrates from cultures were collected for enzymatic activity assay. Means are calculated from three replicates and bars represent standard error.

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**Figure 3. Biomass production of *M. robertsii* 2575 wild-type strain and  $\Delta$ MrInv in the presence of glucose and/or sucrose.** Mycelia were transferred from SDB to minimal liquid medium supplemented with 1% glucose (Glu) and (Glu+Suc)/or 1% sucrose (Suc), grown at 27 °C for 12 h. Means were calculated from 3 replicates with corresponding standard errors. Experiments were repeated twice. \*\* indicates means statistically different at the 0.01 level.

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and *Trichoderma*. Each fungus has evolved its own multifaceted and robust mechanisms to overcome the challenges encountered on plant roots.

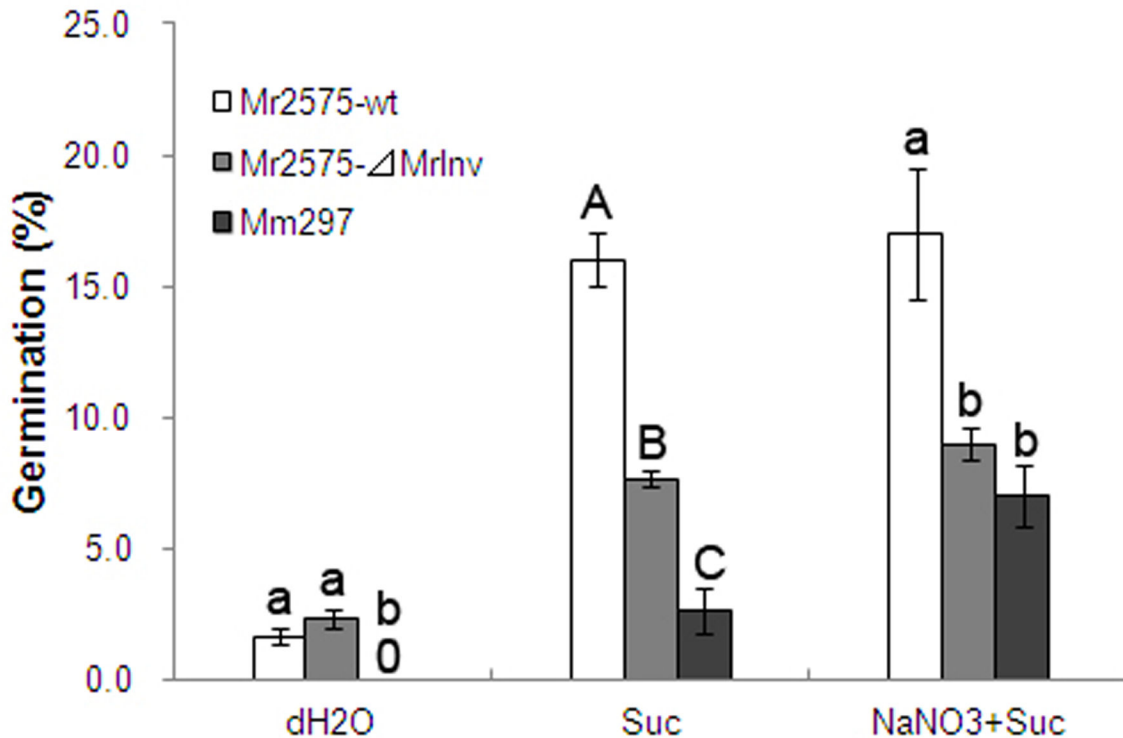
*In vitro* growth assays have shown that *M. robertsii* grows well in root exudates from soybeans and switchgrass [30,32]. In the current study, we show that *M. robertsii* uses MrINV to specifically hydrolyze sucrose to monosaccharides, and while *M. robertsii* utilizes a wide variety of carbohydrates the highest germination rates are on monosaccharides. *M. robertsii* can also take up sucrose using its unique oligosaccharide transporter (MRT) [30,32]. Disruption of *Mrt* resulted in reduced rhizosphere competency confirming a role for root-derived oligosaccharides in the symbiotic relationship between *M. robertsii* and plants [30]. However, germination and growth utilizing sucrose are reduced in the absence of the extracellular sucrose-induced MrINV. Given the complexity of carbon sources in root exudate, diverse strategies for utilizing sugars seems well attuned to *M. robertsii*'s opportunistic lifestyle and wide range of plant associations.

A phylogenetic reconstruction classifies MrINV as a fungal secreted acidic invertase, and it clusters separately from non-secreted enzymes including the query sequence TvINV from *T. virens*. Blastp and genomic blast searches confirm that *MrInv* is a single copy gene in *M. robertsii*. The specialist beetle

pathogen *M. majus* lacks a homolog of MrINV and does not grow on sucrose as sole carbon source. *M. majus* has not to date been identified as a root colonizer [32], suggesting that rhizospheric competence may be related to the sugar metabolism of different *Metarhizium* species.

*Metarhizium* is attracted to and associates intimately with root surfaces in the soil [32,43]. However, few studies have focused on the characteristics of *Metarhizium* interactions with roots as they appear under the microscope. In this study, the microscopic observation of the colonization of *A. thaliana* root by *M. robertsii* suggests the root invasion occurs by penetration of the epidermis and further ingress into the outer cortex, which share a similar pattern of colonization with *Trichoderma* [15]. However, unlike *Trichoderma*, we observed no specific adhesive structures during root colonization by *M. robertsii*. Sasan and Bidochka (2012) have reported that *M. robertsii* promotes plant root growth [43], and we have observed the same in field conditions when plants are growing under sub-optimum feeding and watering regimes (Unpubl. Data). The lack of impact on plant growth in the current study is probably because the plants were growing in a nutrient rich soil with adequate water.

The most significant finding of the current study is that disruption of *MrInv* reduced *M. robertsii*'s survival in the



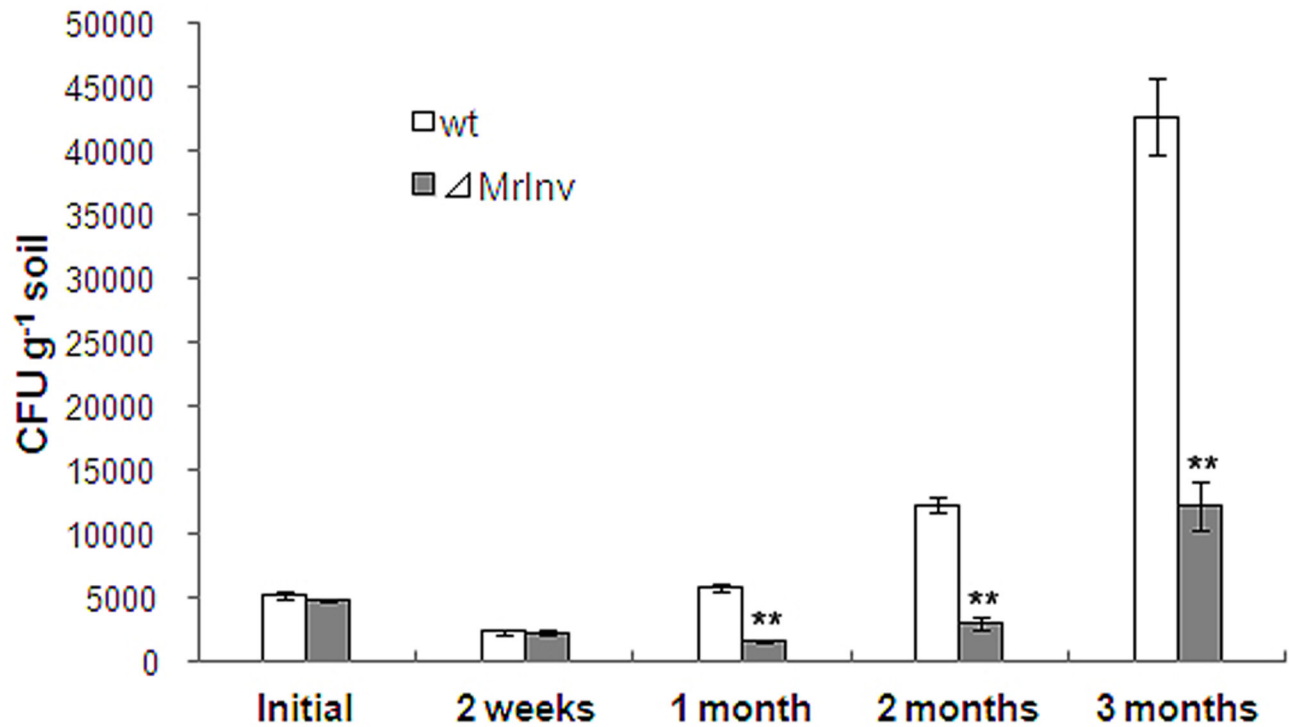
**Figure 4. Conidial germination of *M. robertsii* 2575 wild-type strain (Mr2575-wt), *Mrlnv* disruption mutant (Mr2575- $\Delta$ Mrlnv) and *M. majus* 297 wild-type strain (Mm297).** Conidia were cultured in sterile distilled water (dH<sub>2</sub>O), 0.1% sucrose (Suc) or 0.1% sucrose plus 0.1% NaNO<sub>3</sub> (NaNO<sub>3</sub>+Suc). Conidial germination was determined after 24 h incubation at 27 °C. Means were calculated from 5 replicates. Bars represent the standard error. Upper-case and lower-case letters indicate means statistically different at the 0.01 and 0.05 level.

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overlapping rhizospheres surrounding switchgrass roots, but significantly enhanced root colonization. *Trichoderma* lacking the intracellular *TvInV* also shows increased colonization of maize roots [22]. There is no information about *TvInV*'s effects on competency in soil but *Trichoderma* lacking *TvInV* showed increased expression of hydrolytic enzymes that weaken epidermal cell walls [44]. This could result from carbon catabolite derepression because of a reduction in glucose and related sugars in the mutant [45,46]. The *tps1* (encoding trehalose-6-phosphate synthase) and *hvk1* (encoding hexose kinase) are important mediators of CCR in fungi. They are induced by glucose and mediate glycolysis and carbon catabolite repression [47]. In our study, sucrose induced greater expression of *tps1* and *hvk1* in the wild-type than in  $\Delta$ *Mrlnv* consistent with derepression in the mutant. *M. robertsii* invades insects by direct penetration of the cuticle facilitated by the production of a battery of extracellular enzymes, including

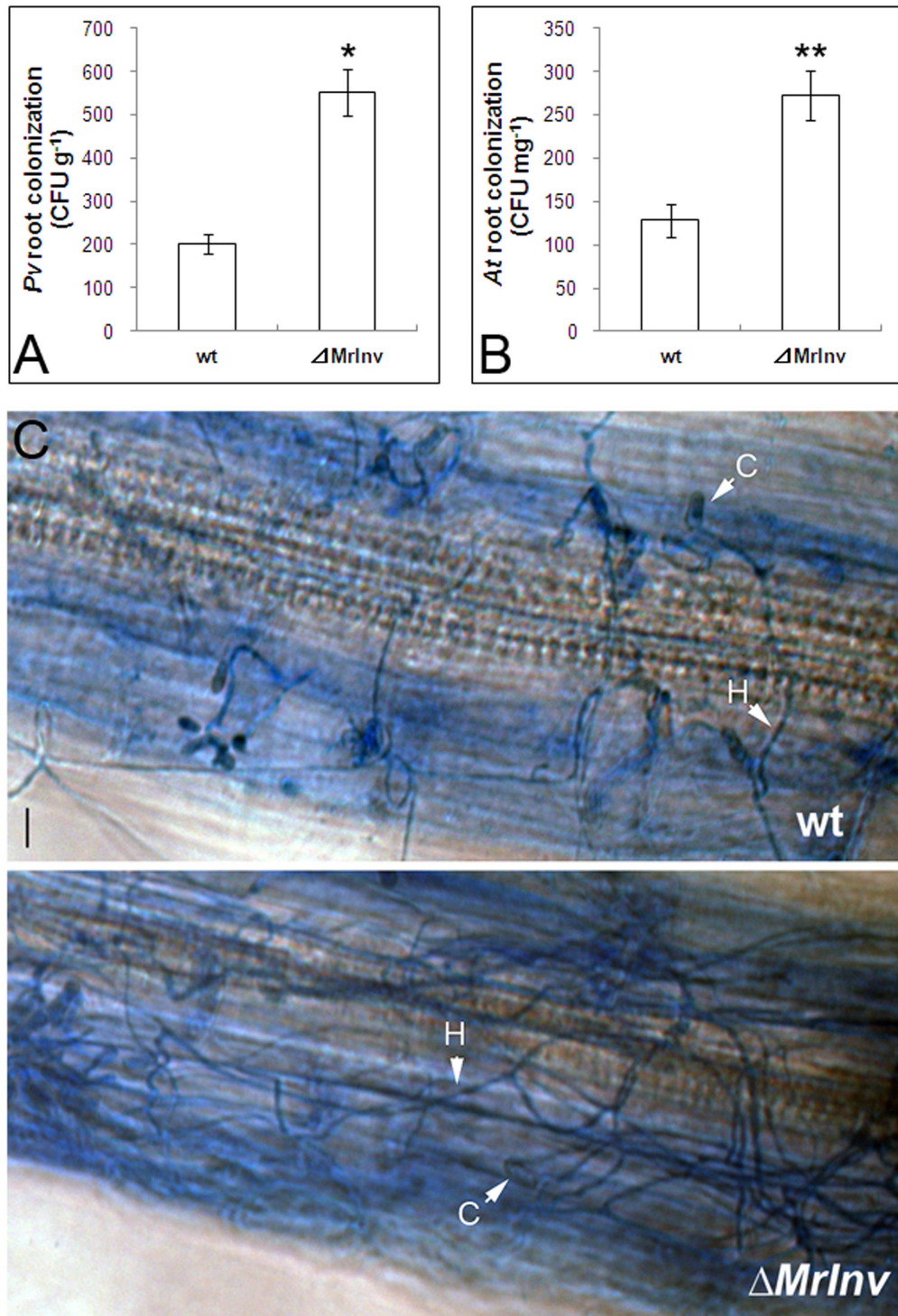
proteinases (Pr1), chitinases and esterases [38]. The higher levels of Pr1 and endochitinase produced by  $\Delta$ *Mrlnv* on *Arabidopsis* root probably result from carbon catabolite derepression in the mutant. In the field these enzymes could be involved in scavenging nutrients from fungi and insects in the soil since they do not target the plant cell wall.  $\Delta$ *Mrlnv*'s upregulation of pectinase, which hydrolyzes a major polysaccharide substrate in plant cell walls [37], provides a direct connection between *Metarhizium*-produced hydrolytic enzymes and plant cell wall-degradation. Furthermore, increased colonization of monocotyledon (switchgrass) and dicotyledon (*A. thaliana*) roots suggests that the effect is irrespective of the plant species. In spite of increased production of cell wall-degrading enzymes  $\Delta$ *Mrlnv*'s competency in the soil is sharply reduced suggesting that at a small distance from the roots sucrose is a more important source of nutrients than polymers.





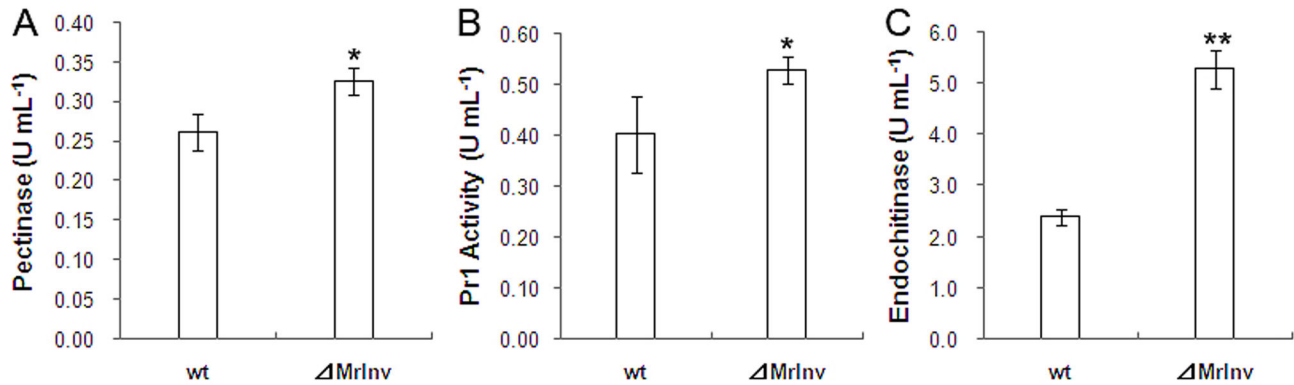
**Figure 5. Rhizosphere competency of *M. robertsii* wild-type strain (wt) and *Mrlnv* disruption mutant ( $\Delta$ Mrlnv).** Switchgrass seeds were inoculated with *M. robertsii* spores and grown in the growth chamber at 25 °C with 14:10 h light:dark cycle. Rhizospheric populations of each strain were measured by counting the number of CFUs in rhizospheric soils. Initial indicates the number of CFUs from each treatment immediately after inoculation. Means were calculated from nine replicates. Bars represent the standard error. \*\* indicates means statistically different at the 0.01 level.

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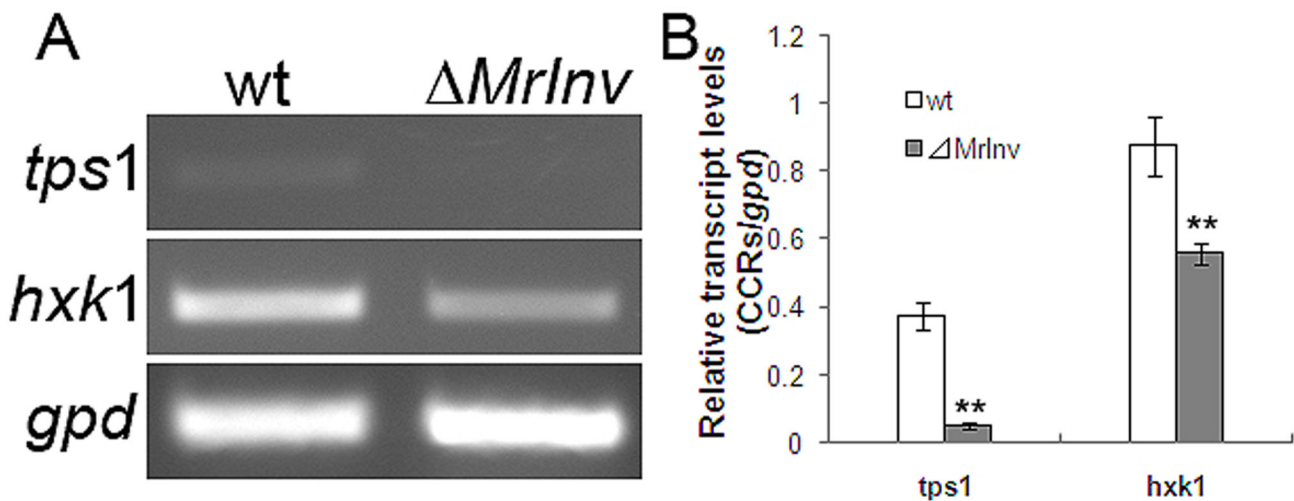
**Figure 6. Root colonization of switchgrass (*Pv*) and *A. thaliana* (*At*) by *M. robertsii* wild-type strain (*wt*) and *MrInv* disruption mutant ( $\Delta$ *MrInv*).** A, 3-month-old switchgrass roots from the soils containing *M. robertsii* strains were collected for root colonization assay. B, *A. thaliana* seedlings were inoculated with *M. robertsii* spores from each strain in the hydroponic system. After 48 h incubation, *A. thaliana* roots were collected for root colonization assay. Means are calculated from 9 (*Pv*) or 27 (*At*) replicates of each treatment and bars represent the standard error. \*\* and \* indicate means statistically different at the 0.01 and 0.05 level. C, Photographs of *A. thaliana* root colonization by *M. robertsii* wild-type strain (top) and  $\Delta$ *MrInv* (bottom) after 48 h incubation. C, conidium; H, hypha. Scale bar = 5  $\mu$ m.

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**Figure 7. The activity assay of hydrolytic enzymes produced by *M. robertsii* 2575 wild-type strain and  $\Delta MrlInv$ .** The filtrates from cultures of each strain grown with *A. thaliana* seedlings for 48 h were collected, and the enzymatic activity of pectinase (A), subtilisin Pr1 (B) and endochitinase (C) were measured. Means are calculated from three replicates and bars represent standard deviation. Similar results were obtained in two independent experiments. \*\* and \* indicate means statistically different at the 0.01 and 0.05 level.

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**Figure 8. Expression of CCR-related genes in *M. robertsii* 2575 wild-type strain and  $\Delta MrlInv$  in the presence of sucrose.** A, Semi-quantitative RT-PCR analysis of CCR-related genes. Mycelia were transferred from SDB to minimal medium plus 1% sucrose, grown at 27 °C for 30 min. Total RNA was extracted from fungal biomass and qPCR was performed. B, Relative transcript levels of CCR-related genes versus the housekeeping *gpd* gene. *tps1*, trehalose-6-phosphate synthase gene; *hxx1*, hexose kinase gene; *gpd*, glyceraldehyde-3-phosphate dehydrogenase gene. Values are means of three replicates with corresponding standard deviation. \*\* indicates means statistically different at the 0.01 level.

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**Table 2.** Commonalities and differences between *Metarhizium* and *Trichoderma* in root colonization.

Genus	<i>Metarhizium</i>	<i>Trichoderma</i>
<b>Pathogen</b>	Of insects	Of fungi (some strains)
<b>Root colonization</b>	Minority of strains	Minority of strains
<b>Beneficial effects</b>	Inhibit plant pathogens [7]	Inhibit plant pathogens [10]
	Enhancing the uptake of micronutrients [8]	Enhancing the uptake of micronutrients [48]
	Translocating nitrogen from killed insects to plants [9]	Facilitate plant resistance to abiotic stress [49]
	Killing soil insects [6]	Induction of plant defenses [44]
		Boosting plant growth by fungus-derived phytohormone [50]
<b>Adhere to root</b>	Adhesin (MAD2) [16]	Hydrophobin [15]
<b>Adhesive structure</b>	No specific adhesive structure	Form chlamydospore [51]
<b>Survival in the soil</b>	Survive adverse environmental conditions [52]	Inability to survive adverse environmental conditions [53]
<b>Nutrient sources</b>	Products of plant photosynthesis, particularly sugars	Products of plant photosynthesis, particularly sugars
<b>Sugars uptake</b>	Raffinose pump (MRT) [30]	
	Invertase (MrINV, <b>this study</b> )	Invertase (TvINV) [22]
<b>INV properties</b>	Acidic	Acidic
<b>INV Localization</b>	Extracellular	Intracellular
<b>INV Loss-of-Function</b>	Impairing rhizosphere competency	
<b>Function</b>	Increase root colonization	Increase root colonization
<b>Mechanism</b>	Upregulation of hydrolytic enzymes through carbon catabolite derepression	Upregulation of hydrolytic enzymes through carbon catabolite derepression

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## Supporting Information

**Figure S1. Verification of *Mrlnv* disruption and complement in *M. robertsii* 2575.** A, The schematic diagram of DNA crossover and integration in the genome of wild-type and mutant strains. B, wt, wild-type stain;  $\Delta Mrlnv$ , the mutant in which *Mrlnv* was replaced with the bar selective marker by homologous recombination;  $\Delta Mrlnv$ -rv, a transformant in which the  $\Delta Mrlnv$  complemented by the *Mrlnv* genomic fragment. (PDF)

**Figure S2. Growth of *M. robertsii* 2575 wild-type strain (wt) and *Mrlnv* disruption mutant ( $\Delta Mrlnv$ ) on minimal medium agar plates supplemented with 1% sucrose at 27 °C for 10 d.  $\Delta Mrlnv$  grew comparatively less well than wild-type strain on sucrose.** (PDF)

**Figure S3. Growth of *M. robertsii* 2575 wild-type strain (wt) and *Mrlnv* disruption mutant ( $\Delta Mrlnv$ ) on PDA (top) for 5 d and M100 (bottom) agar plates at 27 °C for 10 d. Scale bar = 1 cm.**

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(PDF)

**Figure S4. Switchgrass growth was monitored by measuring the shoot length and leaf chlorophyll content at one month intervals post-inoculation.** Switchgrass were harvested after three months. The root length and plant dry biomass were determined. Nine plants from each pot were randomly selected for measurement. Values are means calculated from 27 replicates and bars represent the standard error. (PDF)

**Table S1. Primers used in this study.** (PDF)

## Author Contributions

Conceived and designed the experiments: XL RJSL. Performed the experiments: XL. Analyzed the data: XL WF LL HLL. Contributed reagents/materials/analysis tools: RJSL. Wrote the manuscript: RJSL XL.

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