The small secreted effector protein MiSSP7.6 of *Laccaria bicolor* is required for the establishment of ectomycorrhizal symbiosis

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Summary

To establish and maintain a symbiotic relationship, the ectomycorrhizal fungus *Laccaria bicolor* releases mycorrhiza-induced small secreted proteins (MiSSPs) into host roots. Here, we have functionally characterized the *MYCORRHIZA-INDUCED SMALL SECRETED PROTEIN OF 7.6 kDa* (*MiSSP7.6*) from *L. bicolor* by assessing its induced expression in ectomycorrhizae, silencing its expression by RNAi, and tracking *in planta* subcellular localization of its protein product. We also carried out yeast two-hybrid assays and bimolecular fluorescence complementation (BiFC) analysis to identify possible protein targets of the MiSSP7.6 effector in *Populus* roots. We showed that *MiSSP7.6* expression is upregulated in ectomycorrhizal rootlets and associated extramatrical mycelium during the late stage of symbiosis development. RNAi mutants with a decreased *MiSSP7.6* expression have a lower *mycorrhization rate,* suggesting a key role in the establishment of the symbiosis with plants. MiSSP7.6 is secreted and it localizes both to the nuclei and cytoplasm in plant cells. MiSSP7.6 protein was shown to interact with two *Populus* Trihelix transcription factors. Furthermore, when co-expressed with one of the Trihelix transcription factors, MiSSP7.6 is localized to plant nuclei only. Our data suggest that MiSSP7.6 is a novel secreted symbiotic effector and is a potential determinant for ectomycorrhiza formation.

Key words: Ectomycorrhizal fungus, effector, *Laccaria bicolor*, small-secreted protein, Trihelix transcription factors

Introduction

In temperate and boreal forest ecosystems, most tree species form ectomycorrhizal (ECM) symbiosis with soil fungi in Ascomycota and Basidiomycota (Tedersoo & Smith, 2013). Upon symbiosis, host plants supply photosynthetically-derived carbohydrates to the fungi in exchange for N and Pi from soils, and this process is critical for the C and N cycles in boreal and temperate forests (Van der Heijden *et al*., 2015). Besides their critical ecological roles, several ECM plants are timber species, while dozens of ECM fungi can produce edible mushrooms, a valuable resource in rural areas worldwide (Mello & Balestrini, 2018).

However, unlike the legume-rhizobia and arbuscular mycorrhizal symbioses whose molecular mechanisms have been studied in details (Oldroyd, 2013), the molecular definition of ectomycorrhizae development remains a major challenge (Martin *et al*., 2016). A few symbiosis-related genes have however been functionally characterized recently (Plett *et al.*, 2011, 2014; Pellegrin *et al.*, 2019; Vayssières *et al*., 2015; Zhang *et al*., 2018) by using the *Laccaria-Populus* mutualistic association. The genome from the basidiomycete *Laccaria bicolor* encodes 300+ lineage-specific small secreted proteins (<300 amino acids with a predicted signal peptide) (Martin *et al*., 2008). Transcript profiling showed that the expression of more than 50 genes coding for small secreted proteins is strikingly induced in ectomycorrhizae and these proteins were named Mycorrhiza-induced Small Secreted Proteins (MiSSPs) (Martin *et al*., 2008). They are candidate effector proteins which can either translocate into specific compartments of the host cells (Plett *et al.*, 2011) or reside in the symbiotic interface (Pellegrin *et al*., 2019). MiSSPs lack known functional domains and only a subset is conserved in related ectomycorrhizal fungi (Kohler *et al.*, 2015).

Functional analyses, including loss-of-function approaches, are needed to determine whether *L. bicolor* MiSSPs that have been inferred from symbiotic transcriptomes (Martin *et al*., 2008; Kohler *et al*., 2015; Plett *et al*., 2015) contribute to symbiosis by targeting host regulatory proteins and/or participating in the construction of the novel symbiotic apoplastic interface. Up to date, only two effectors, MiSSP7 and MiSSP8, have been functionally characterized (Plett *et al*., 2011, 2014; Pellegrin *et al*., 2019).

MYCORRHIZA-INDUCED SMALL SECRETED PROTEIN OF 7 KDA (MiSSP7) and *MYCORRHIZA-INDUCED SMALL SECRETED PROTEIN OF 8 KDA* (*MiSSP8)* are among the *L. bicolor MiSSP*-encoding genes displaying the highest upregulation in ectomycorrhizal rootlets (Martin *et al*., 2008). *MiSSP7* expression is induced by the plant-derived flavonoid rutin (Plett and Martin, 2012) and codes for a small secreted protein of 7 kDa released into the symbiotic interface, imported into host plant cells and translocated to root cell nuclei within a few minutes, where its accumulation rapidly alters gene expression (Plett *et al*., 2011). Furthermore, *L. bicolor MiSSP7* RNAi lines with lower *MiSSP7* expression are no longer able to form the intraradical Hartig net on its poplar host roots (Plett *et al*., 2011). In plant nuclei, MiSSP7 interacts with the transcriptional repressor Jasmonate Zim Domain protein 6 (JAZ6) to modulate the jasmonate signaling pathway and dampen the plant defense reactions (Plett *et al*., 2014). On the other hand, MiSSP8 from *L. bicolor* is a small secreted protein containing a DWRR repetitive motif, which accumulates in ectomycorrhizal rootlets and fruiting bodies. These repeats are likely processed by the Kex2 protease to release the DSDWR peptide that might play a role in hyphae aggregation. The *MiSSP8*-RNAi knockdown mutants are strongly impaired in their ability to establish ectomycorrhizae (Pellegrin *et al*., 2019). These studies suggest that several MiSSPs may have a key role in the rewiring of host plant

signalling that is required to enable ectomycorrhizal associations to develop and efficiently function.

In this study, we functionally analyze the secreted effector *MYCORRHIZA-INDUCED SMALL SECRETED PROTEIN OF 7.6 KDA (MiSSP7.6)* of *L. bicolor* and provide evidence that this effector interacts with immunity-related transcription factors in *Populus*.

Results

MiSSP7.6 is secreted as indicated by the yeast invertase secretion assay

As a mycorrhiza-induced secreted protein, MiSSP7.6 belongs to the *L. bicolor* candidate effector proteins (Martin *et al.*, 2008; Kohler *et al.*, 2015; Plett *et al*., 2015). MiSSP7.6 is predicted to be secreted and the mature polypeptide contains 54 amino acids (pI of 3.91, MM of 5454.16 Da), without any predicted known functional or structural domains (Fig. 1A). A BLAST query at GenBank and MycoCosm databases identified a single orthologous protein, MiSSP7.6 from *Laccaria amethystina* (JGI ID #677959), indicating that MiSSP7.6 is a clade-specific protein of unknown function. The putative secretory signal peptide of MiSSP7.6 (Supporting Information Fig. S1) is functional in yeast as shown by the yeast invertase secretion assay (Fig. 1B).

MiSSP7.6 expression is induced during ectomycorrhizae formation

Genome-wide transcript profilings showed that *MiSSP7.6* expression is up-regulated >100-fold in *P. trichocarpa-L. bicolor* ectomycorrhizal rootlets (Martin *et al*., 2008; Plett *et al.*, 2015). The qRT-PCR analysis showed that *MiSSP7.6* was barely detectable in axenic culture of *L. bicolor*, but was highly induced in ectomycorrhizal root tips of

L. bicolor–P. tremula x alba. This induction was already observed two weeks post contact when hyphae form the mantle sheath (Fig. 2) and *MiSSP7.6* expression peaked at four weeks post contact when the Hartig net is fully developed. *MiSSP7.6* was also highly induced in running hyphae attached to ectomycorrhizal rootlets (so-called extramatrical mycelium), indicating that direct physical contact with roots is not required to trigger *MiSSP7.6* induction (Fig. 2).

Impairment of the mycorrhiza formation rate by RNAi-mediated knockdown of MiSSP7.6

To investigate the potential contribution of MiSSP7.6 to ectomycorrhizae formation, we produced several transgenic *L. bicolor* lines expressing *MiSSP7.6* RNAi construct or the empty vector control. qPCR assay showed that *MiSSP7.6* relative expression in RNAi transformants decreased by 75% to 90% as compared to empty vector control lines (Fig. 3A). No obvious growth defect was observed for these *MiSSP7.6* RNAi lines as compared to empty vector controls (Supporting Information Fig. S2). The ability of *MiSSP7.6* RNAi lines to colonize *P. tremula* x *alba* roots was assessed after three weeks of co-culture. The proportion of ectomycorrhizal rootlets was significantly reduced and dropped from approx. 60% to 20% for *MiSSP7.6* RNAi lines ($P < 0.05$, $n = 36$) when compared to empty vector control lines (Fig. 3B). Next, we investigated root colonization by *MiSSP7.6-RNAi* lines by CLSM and compared this to colonization by the empty vector control lines (Fig. 3C). The fungal mantle did not seem to be reduced, but a reduced intraradical Hartig net was observed as compared to empty vector controls. These results indicate that down-regulation of *MiSSP7.6* gene expression by RNAi has a negative effect on ECM formation.

Subcellular localization of MiSSP7.6 in plant cells

We localized MiSSP7.6 in *Nicotiana benthamiana* leaf epidermal cells expressing EGFP fused to either a full-length MiSSP7.6 or MiSSP7.6 without its signal peptide (MiSSP7.6ΔSP) under the control of the constitutive CaMV 35S promoter. The EGFP-tagged MiSSP7.6 was mainly localized in the cytosol of tobacco cells (Supporting Information Fig. S3A), whereas MiSSP7.6ΔSP also accumulated strongly in nuclei (Supporting Information Fig. S3B), suggesting that MiSSP7.6 might translocate to the root cell nuclei after its secretion.

Subcellular localization of MiSSP7.6 in L. bicolor

We determined the subcellular localization of MiSSP7.6 in *L. bicolor*. The cDNA encoding MiSSP7.6, with or without signal peptide, was fused to EGFP in frame and expressed with *Agaricus bisporus GPD* promoter. No significant fluorescence was observed in axenic hyphae (data not shown). It has been reported that intron splicing is required for efficient heterologous gene expression in Basidiomycota (Al-Salihi *et al.*, 2017). Therefore, we used the genomic, intron-containing gene sequence of *MiSSP7.6* to express the EGFP fusion protein. For the *MiSSP7.6* gene, including the signal peptide sequence, no fluorescence was detected. In contrast, a strong fluorescence accumulated throughout transgenic hyphae for the *MiSSP7.6* construction without the signal peptide sequence (Fig. 4A). To validate our transformation protocol, we assessed the localization of the *L. bicolor* histone *H2B* gene (HTB16202; JGIv2 ID # 185040) fused to EGFP (Kemppainen and Pardo, 2017). The H2B-EGFP strongly accumulated into nuclei (Fig. 4B).

MiSSP7.6 interacts with Trihelix transcription factors

Having shown that MiSSP7.6 is likely secreted in the extracellular medium and might translocate to nuclei of poplar root cells, we speculated interaction(s) of MiSSP7.6 with the host nuclear proteins. To identify the interaction partners from *P. tremula x alba*, we used YTH assay and pooled cDNA libraries of *L. bicolor–P. trichocarpa* ectomycorrhizal roots (Plett *et al*., 2014). Positive interaction clones from the primary screens (SC-W-L-H+3AT) were tested on a stringent selective medium (SC-W-L-U). The false positive interactions were further eliminated by reverse YTH by 5FOA reverse selection (Fig. 5). Screening with MiSSP7.6 lacking its signal peptide as a bait retrieved two different proteins annotated as Trihelix transcription factor GT-3b in the *P. trichocarpa* JGI Phytozome database. They belong to the Zinc-Finger superfamily and the Myb/SANT-like DNA-binding domain protein family. These two proteins, named PtTrihelix1 (Potri.001G129900) and PtTrihelix2 (Potri.001G066900) (Fig. S4), share 17% amino acid identity (Supporting Information Fig. S4C), but both proteins display the typical trihelix DNA binding domain at their N-terminus containing three amphipathic α-helices and the conserved tryptophan (W) residues (Riechmann *et al*., 2000). The predicted coiled-coil domain at the C-terminus (Lupas *et al*., 1991) is loosely conserved (Supporting Information Fig. S4C). Interestingly, the closest homolog of PtTrihelix2 is the ASR3 (ARABIDOPSIS SH4-RELATED3) protein (Supporting Information Fig. S4C), which is a phosphorylation substrate of MAP KINASE4 and negatively regulates *Arabidopsis* immunity (Li *et al*., 2015). The two genes, *PtTrihelix1* and *PtTrihelix2*, are expressed at similar level in non-mycorrhizal and ectomycorrhizal roots (11 to 28 RPKM) and they are not differentially expressed during ectomycorrhizae formation (data not shown).

To confirm the specificity of these protein interactions, a point-to-point YTH assay was performed between PtTrihelix1, PtTrihelix2, and an unrelated protein Krev1 was used as YTH assay control. Neither PtTrihelix1 and PtTrihelix2 interacted with Krev1 (Fig. 5), indicating that the interactions were specific in yeast and that the two PtTriHelix proteins do not autoactivate the reporter genes. The interaction strength was measured through the ß-galactosidase activity on plates containing X-Gal. Yeast clones showing blue color suggest a relative strong interaction, indicating that the interaction between PtTrihelix1 and MiSSP7.6 was stronger than interaction between PtTrihelix2 and MiSSP7.6 (Fig. 5).

Interaction of PtTrihelix1, PtTrihelix2 and MiSSP7.6 in planta

To validate the interaction between PtTrihelix1, PtTrihelix2 and MiSSP7.6, we performed bimolecular fluorescence complementation (BiFC) assays. We constructed plasmids that would express a fusion protein of the C-terminal split cyan fluorescent protein (SCFP) (Waadt et al., 2008; Gehl et al., 2009) with PtTrihelix1 and PtTrihelix2 (SCFPC-PtTrihelix1 and SCFPC-PtTrihelix2) and a fusion protein of MiSSP7.6 and MiSSP7.6ΔSP (MiSSP7.6 without signal peptide) with the N-terminal Venus (nVenus-MiSSP7.6 and nVenus-MiSSP7.6ΔSP). The constructs were transiently co-expressed in *N. benthamiana* epidermal cells. In the co-expression of MiSSP7.6 or MiSSP7.6ΔSP with PtTihelix1 we observed strong fluorescence signals in the nucleus (Fig. 6A, B). However, for PtTrihelix2, the interactions were observed only for MiSSP7.6ΔSP (Fig. 6D) but not MiSSP7.6 (Fig. 6C). These data suggested that the interaction pattern between PtTihelix1 and MiSSP7.6 was, to some extent, different from the interaction between PtTihelix2 and MiSSP7.6.

PtTrihelix1 is able to form homodimers

We assessed the ability of PtTrihelix1 and PtTrihelix2 to assemble in homo- or hetero-dimers by using the BiFC assay. Co-expression of SCFPC-PtTrihelix1 and nVenus-PtTrihelix1 resulted in high fluorescence signals in tobacco nuclei (Fig. 6E), suggesting the formation of a homodimer. On the other hand, no fluorescence signal was detected for PtTrihelix2/PtTrihelix2 (data no shown) or PtTrihelix2/PtTrihelix1 co-expressions (Fig. 6F).

PtTrihelix1, but not PtTrihelix2, changes MiSSP7.6 localization in tobacco cells

We assessed whether PtTrihelix1 or PtTrihelix2 could change MiSSP7.6 localization when co-expressed in *N. benthamiana* leaf cells. MiSSP7.6 was thus fused to EGFP, while PtTrihelix1 and PtTrihelix2 were fused to RFP under the control of 35S promoter. When co-expressed in leaf epidermal cells, EGFP-MiSSP7.6 and RFP-PtTrihelix1 co-localized in nuclei (Fig. 7A) and accumulation of EGFP-MiSSP7.6 in the cytosol strongly diminished. Note that the localization of the EGFP protein alone did not change when co-expressed with RFP-PtTrihelix1(Fig. 7B). EGFP-MiSSP7.6 and RFP-PtTrihelix2 were also found to co-localize in the nuclei, but the cytoplasmic accumulation of EGFP-MiSSP7.6 did not changed (Fig. 7C), suggesting that the interaction between MiSSP7.6 and PtTrihelix1 or PtTrihelix2 is not strictly identical.

Discussion

Genome-wide transcript profiling of a dozen of mycorrhizal associations have revealed that 7 to 38% of the fungal genes that are upregulated during symbiosis are species-specific genes (Kohler *et al.*, 2015; Martin *et al.*, 2016). Among these symbiosis-induced

genes, 8 to 28% encode candidate secreted effectors (less than 300 amino acids with a predicted signal peptide and no known function) (Kohler *et al.*, 2015) that were named mycorrhiza-induced small secreted proteins (MiSSPs) (Martin *et al*., 2008). MiSSP expression is modulated by various environmental factors (Doré *et al.*, 2017) and the plant host identity (Plett *et al*., 2014).

Among the >50 MiSSPs encoded by *L. bicolor*, only two have been functionally characterized so far, MiSSP7 and MiSSP8. *MiSSP7* is the most highly upregulated fungal gene in *P. trichocarpa* ectomycorrhizal root tips (Martin *et al*., 2008), and it was the first gene shown to be required for the development of the ectomycorrhizal symbiosis (Plett *et al*., 2011). In the host nucleus, MiSSP7 interacts with the transcriptional repressor JASMONATE ZIM DOMAIN protein 6 (JAZ6), which is a key regulator of the jasmonate signalling pathway (Plett *et al*., 2014). Thus, *L. bicolor* uses MiSSP7 to manipulate a major hormone receptor and related signalling pathways of its host to facilitate its root ingress while escaping plant defense reactions. *MiSSP8* is also highly upregulated in symbiotic tissues. Silencing of *MiSSP8* by RNAi also prevents the Hartig net development (Pellegrin *et al.*, 2019). Besides ectomycorrhizal rootlets, *MiSSP8* also accumulates in fruiting bodies, which is something different from MiSSP7 (Pellegrin *et al.*, 2019). Interestingly, some of small secreted proteins (SSPs) have recently been shown to be upregulated during fruiting bodies development in the Schizophyllaceae by comparative genomic and transcriptomic analyses (Almasi et al., 2019).

Here, we demonstrate that the secreted effector MiSSP7.6 of *L. bicolor* is an additional symbiotic factor controlling ectomycorrhizae development in *Populus*. MiSSP7.6 is a lineage-specific cysteine-rich protein only found in *L. bicolor* and its sister species *L. amethystina*. Reduction of *MiSSP7.6* expression by RNAi restricts ectomycorrhizae

establishment in *P. tremula x alba* and prevents the development of the intraradicular hyphal Hartig net. MiSSP7.6 is secreted and localized to the plant cell cytosol and nuclei. As shown by Y2H and BiFC assays, MiSSP7.6 translocated to the host nuclei can interact with the Trihelix transcription factors, PtTrihelix1 and PtTrihelix2. Both of these transcriptional regulators contain a N-terminal trihelix DNA binding domain and a C-terminal coiled-coil domain. PtTrihelix2 shows its closest sequence similarity to ASR3 from *Arabidopsis*. Phosphorylation of ASR3 by MAP KINASE4 negatively regulates pattern-triggered immunity in *Arabidopsis thaliana* (Li *et al*., 2015). We speculate that MiSSP7.6 is expressed by *L. bicolor* upon contact with poplar roots, secreted into the apoplastic symbiotic interface and translocated to the host cells. There, MiSSP7.6 binds to PtTrihelix regulators to fine-tune the plant immune gene expression during *L. bicolor* ingress. Together with MiSSP7, which dampens the plant defense reaction by modulating the jasmonate pathway signalling, MiSSP7.6 may alter the host plant immunity.

In summary, we have functionally characterized a new mycorrhiza induced small secreted protein *MiSSP7.6* in *L. bicolor* during ectomycorrhizal symbiosis establishment. We also identified two host plant PtTrihelix type transcription factors as interacting targets of MiSSP7.6. The mechanism of PtTrihelix action in *Populus* is unknown and will require identification of the binding sites driving the MiSSP7.6/PtTrihelix interaction, as well as characrterization of PtTrihelix binding partners in symbiotic roots. Moreover, the role of PtTrihelix transcription factors in poplar immunity needs to be confirmed in future research.

Experimental procedures

Biological materials and growth conditions

The free-living mycelium of the S238N strain of *L. bicolor* (Maire) P.D. Orton was grown at 24 °C on modified Pachlewski agar medium P5 (Felten *et al*., 2009). Cuttings of the hybrid grey poplar (*Populus tremula*× *alba* (synonym *Populus canescens*) INRA clone 717-1B4) were micropropagated *in vitro* and grown on half Murashige and Skoog (MS) medium in glass culture tubes under a 16-h light/8-h dark photoperiod at 24 °C and 70% relative humidity in a growth chamber. Wild-type *Nicotiana benthamiana* plants were grown from seeds in a growth chamber at 22 °C and 70% relative humidity under a 16-h light/8-h dark photoperiod for 1 to 1.5 months before infiltration with *Agrobacterium tumefaciens*.

In vitro formation of ectomycorrhizae

Ectomycorrhizae were established *in vitro* according to the "sandwich" co-culture system (Felten *et al*., 2009). Two-centimeter long poplar cuttings were rooted on solid agar MS medium for three weeks under a 16-h light/8-h dark photoperiod at 24 °C and 70% relative humidity in a growth chamber. Mycelial cultures of *L. bicolor* S238N (or transformed RNAi lines) were grown for 10 days on Pachlewski P5 agar medium in the dark on cellophane membranes placed on the agar surface. Then, membranes with *L. bicolor* mycelium and poplar seedlings were transferred onto the surface of low glucose Pachlewski agar medium (0.1% glucose) containing 0.1% MES (Sigma), and covered by another cellophane membrane. After two, three and four weeks of co-culture, the percentage of mycorrhizal roots was assessed. For each sampling, five replicates were

analyzed. Each replicate consisted of one dish containing three plantlets, and 25 to 50 ectomycorrhizal root tips from these three plantlets were pooled into a single replicate.

Gene expression analysis by qPCR

The expression profile of the *MiSSP7.6* gene (JGIv2 ID #617208) was analyzed by quantitative RT-PCR using total RNA extracted from free-living axenic mycelium, ectomycorrhizal rootlets at 2-, 3-, and 4-week post-contact, and extramatrical mycelium (i.e., running hyphae attached to ectomycorrhizla rootlets) at 2-, 3-, and 4-week post-contact. Total RNA was isolated using RNeasy Plant Kit (Qiagen, France) according to the manufacturer's instructions and treated with DNase I (Qiagen) to eliminate any genomic DNA contamination. The amount of total RNA was normalized by measuring RNA concentration at 260 nm and 1 µg of total RNA was used for first-strand cDNA synthesis with oligo(dT) primer. All primers were ordered from Eurogentec (Angers, France) and PCR amplification was performed using *Taq* DNA Polymerase (Thermo Fisher Scientific, France) according to the manufacturer's instructions and optimized according to each primer pairing. Real-time PCR analyses were performed using the Fast SYBR Green Master Mix (Applied Biosystems, France) with a final concentration of 0.3 µM of each primer following the manufacturer's instructions. The thermal-cycling condition parameters of the StepOnePlus System qPCR apparatus (Applied Biosystems, France) were as follows: 95°C for 3 min; 40 cycles of 95°C for 15 s, 60°C for 30 s followed by a melting curve. PCR amplifications were carried out on three biological replicates and included two distinct technical replicates. Transcript abundance was normalized using two constitutively expressed *L. bicolor* genes coding for a histone H4 (JGIv2 ID# 319764) and ubiquitin (JGIv2 ID# 446085). Primer sequences were designed

using the nucleotide sequences retrieved from the *L. bicolor* genome v2.0 (http://genome.jgi.doe.gov/Lacbi2/Lacbi2.home.html) and available online tools (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Transcript abundance was quantified using the standard curve method of quantification (based on ΔΔCt calculations), as previously described by Pfaffl (2001). For all real-time qPCR experiment, three biological and two technical repeats were performed. A Student's t test was used to determine the significance ($p < 0.05$) of all results.

Yeast secretion trap assay

Functional analysis of the predicted signal peptide of MiSSP7.6 was done using yeast secretion trap assay with a Gateway-compatible vector pSUC2-GW (Plett *et al.,* 2011). The nucleotide sequences of *MiSSP7.6* with or without its signal peptide were cloned into pSUC2-GW plasmid carrying the yeast invertase, *SUC2*, lacking its native signal peptide. Yeast strain YTK12 was transformed using the lithium acetate method (Gietz and Schiestl, 2007). All transformants were selected on yeast minimal medium without tryptophan. To assess invertase secretion, overnight yeast cultures were diluted to $A_{600} = 1.0$ and a 20 µL-aliquot was plated onto YPSA medium (1% yeast extract, 2% peptone, 2% sucrose, and 1 $\mu g.mL^{-1}$ antimycin , an inhibitor of cytochrome c oxidase). The YTK12 strains transformed with the pSUC2-GW empty vector were used as negative control, and the yeast invertase with signal peptide or signal peptide alone were used positive controls.

Genetic transformation of Laccaria bicolor

Ten independent lines of *L. bicolor* S238N with silenced *MiSSP7.6* expression were created using RNAi (Kemppainen and Pardo, 2010). The full-length coding region of *MiSSP7.6* was amplified by PCR and ligated as inverted repeat into pSILBAγ vector (Kemppainen and Pardo, 2010). This intron-hairpin RNA (ihpRNA) expression construct, which in the fungus is transcribed from *Agaricus bisporus* glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter, was further cloned as a full length SacI-linearized pSILBAγ plasmid into the T-DNA of the binary vector pHg (Kemppainen and Pardo, 2010) to create pHg/pSILBAγTMiSSP7.6. Completed ihpRNA expression cassette was further cloned as a full length SacI-linearized pSILBAγ plasmid into the T-DNA of the binary vector pHg to create pHg/pSILBAγTMiSSP7.6. This RNAi binary vector was transferred into *A. tumefaciens* strain AGL1 by electroporation and was used for transformation of the dikaryotic strain S238N (Kemppainen *et al*., 2005). The transformed fungal strains were selected with 300 $\mu g.mL^{-1}$ hygromycin B (Invitrogen) and were later maintained under selection pressure by using 150 $\mu g.mL^{-1}$ hygromycin B on modified P5 medium (Kemppainen *et al*., 2005). Ten randomly selected pHg/pSγMiSSP7 *L. bicolor* transformant strains were used for further molecular and physiological analyses. Three independent empty vector transformant lines were also phenotyped and they established ectomycorrhizae similarly to the wild-type strain. The growth rate of mutant and wild-type strains was measured on Pachlewski agar medium by inoculating the center of a Petri dish with 0.4 x 0.4 cm agar plug of each fungal strain. The diameter of the fungal colonies was measured every 10 days for 30 days for five biological replicates.

Microscopy and measurements of Hartig net development

Ectomycorrhizal rootlets were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C for 24 h. Infiltration of the fixative was facilitated by placing the samples under vacuum for 1 h. Following fixation, samples were washed twice in 1×PBS (pH 7.4), and were then embedded in 5 % (w/v) agarose. Sections (25 µm) of ectomycorrhizal roots were cut using a VT1200S vibratome (Leica Microsystems, Nanterre, France) and pooled according to their position along rootlets (100, 200 or 600 µm from the root apex). Sections were incubated in 10 µg ml^{-1} wheat germ agglutinin (WGA)–Alexa Fluor 488 conjugate (W21404, ThermoFisher, France) and 1 µg ml^{-1} propidium iodide (Invitrogen, France). To compare the development of the Hartig net between samples, sections from 10 to 15 ectomycorrhizal rootlets (from three replicates), collected at 200 or 600 µm from the root apices, were measured. The Hartig net development was assessed in triplicate by measuring the hyphal ingress between rhizodermal cells on microscopy images using ImageJ program (Schneider *et al*., 2012)).

Cell imaging by confocal laser scanning microscopy

Transversal sections of ectomycorrhizal rootlets were observed using Zeiss LSM780 confocal laser scanning microscope (CLSM) (Carl Zeiss AG, Germany). Images were obtained with the CAPO-40x/1.2 water-immersion objective, acquired sequentially to exclude excitation and emission crosstalk (when required). Spectral deconvolution was used to assess the specificity of emission signal. WGA-AlexaFluor488 and iodure propidium were excited at 488 and 561 nm, respectively, and their specific emission signals were collected between 500-550 nm and between 600-700 nm, respectively. Acquired images were processed using the CLSM ZEN software (Carl Zeiss AG).

Screening of yeast two-hybrid libraries

Yeast two-hybrid assays were carried out as described (Walhout and Vidal, 2001; Plett *et al*., 2014). Bait MiSSP7.6 protein was fused in-frame with the *GAL4* DNA-binding domain in the bait vector pDEST32 (Invitrogen) with Gateway recombination and was transformed into yeast strain MaV103 by the lithium acetate method (Gietz and Schiestl, 2007). The cDNA library was fused to the *GAL4* activation domain in pDEST22, and was transformed into yeast strain MaV203. Mating of MaV103 and MaV203 was performed with MiSSP7.6 as bait protein against a cDNA library of *P. trichocarpa r*oots colonized with *L. bicolor* at different stages of development (Plett *et al.*, 2014). The activity of the yeast β-galactosidase, a subunit of which was coded by the *GAL*1::*lac*Z reporter, was tested according to a previously published method (Walhout and Vidal, 2001).

Bimolecular fluorescence complementation (BiFC) analysis

The cDNAs of *MiSSP7.6*, *TRIHELIX1* and *TRIHELIX2* were amplified by PCR and cloned into the pDONR207 (Invitrogen) using the Gateway BP-reaction to create entry clones, with and without the final stop codon. These entry clones in combination with the appropriate destination vectors: p(MAS)DEST-SCYCE(R)^{GW}, pDEST-VYVE(R)^{GW} (Waadt *et al*., 2008; Gehl *et al*., 2009) were used to create the final Gateway-expression constructs by LR-reaction. To assess the interaction of PtTrihelix1, PtTrihelix2 and MiSSP7.6 polypeptides *in planta*, we also constructed plasmids that express a fusion protein of the C-terminal split cyan fluorescent protein (SCFP) (Waadt *et al*., 2008; Gehl *et al*., 2009) with PtTrihelix1 and PtTrihelix2 (SCFPC-PtTrihelix1 and SCFPC-PtTrihelix2) and a fusion protein of MiSSP7.6ΔSP (MiSSP7.6 without signal peptide)

with the N-terminal Venus (nVenus-MiSSP7.6ΔSP). All expression constructs were transferred into *A. tumefaciens* strain GV3101 by electroporation. Transient expression of proteins in *N. benthamiana* leaves by *A. tumefaciens*-infiltration was conducted as described in Kang *et al*. (2014). Fluorescence of the lower epidermis of leaf discs was assayed three to five days after infiltration using Zeiss LSM780 CLSM.

Subcellular localization of MiSSP7.6 protein in L. bicolor *hyphae*

The binary vector pHyg-EGFP, based on pBGgHg (Chen *et al*., 2000) was constructed for studying the subcellular localization of MiSSP7.6 in *L. bicolor*. The expression cassette was amplified by PCR and inserted into the plasmid using In-Fusion HD Cloning Plus Kit (Clontech). The cDNA encoding Enhanced Green Fluorescent Protein (EGFP) with *Aspergillus nidulans trpC* terminator was placed behind *A. bisporus GPD* promoter (Kemppainen and Pardo, 2010). The whole expression unit was then cloned into the BamHI/SacI site of *pBGgHg* to generate *pHyg-EGFP*, which eliminated the multiple cloning site and introduced new multiple cloning sites in front of EGFP.

The *MiSSP7.6* cDNA or genomic DNA (gDNA, with introns) without the stop codon were cloned into the corresponding cloning sites of *pHyg-EGFP* to obtain *MiSSP7.6*-cDNA-*EGFP* and *MiSSP7.6*-gDNA-*EGFP* fusions. These constructs were transformed into *A. tumefaciens* strain AGL1 for *L. bicolor* transformation. Fluorescence signals of transformed hyphae were examined using a Zeiss LSM780 CLSM.

*Subcellular localization of MiSSP7.6 and PtTrihelix proteins in tobacco cell*s

The mature version of *MiSSP7.6* without signal peptide and without stop codon was amplified by PCR and cloned into *pDONR207* (Invitrogen) using the Gateway BP-

reaction to produce entry clones. The entry clone in combination with the destination vector *pK7WGF2* or *pK7WGR2* (Karimi *et al*., 2002) was used to construct the final Gateway-expression constructs by LR-reaction, i.e. EGFP tag fused at the N-terminal of MiSSP7.6 protein, and RFP tag fused at the N-terminal of PtTrihelix1 or PtTrihelix2 protein. The subcellular localization of EGFP or RFP fusion proteins were examined using a Zeiss LSM780 CLSM.

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Author contributions

HK and FMM planned and designed the project. HK, XC and MK performed the experiments. HK, CV-F, AK, AGP analyzed the data. HK and FMM wrote the manuscript with help of CV-F and MK.

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Figure legends

Fig. 1. MiSSP7.6 signal peptide is functional in yeast as shown by the yeast invertase secretion assay. A. The protein sequence of MiSSP7.6 with the predicted secretory signal peptide showed in bold. The mature peptide of 54 amino acids is rich in cysteine (red) and proline (blue) residues. B. Yeast strain YTK12 was transformed with *MiSSP7.6* with (MiSSP7.6:Invertase) or without signal peptide (MiSSP7.6Δ1-20:Invertase), and grown on medium containing sucrose with or without antimycin. The full-length yeast invertase (SUC2:Invertase) and signal peptide alone (SP_{SUC2}:Invertase) alone was used as a positive control. The empty vector pSUC2-GW was used as a negative control.

Fig. 2. Expression of *MiSSP7.6* gene from *L. bicolor* is induced during ectomycorrhizae development in *P. tremula x alba*. Quantification of *MiSSP7.6* transcripts in ectomycorrhizal roots (ECM) of *P. tremula x alba* 2, 3 and 4 weeks after contact compared to the root-associated extramatrical mycelium (ExM) and axenic free-living mycelium (FLM). Error bars represent ± standard deviation from three biological replicates. Stars indicate significant differences (p-value cutoff < 0.05) from FLM using Welch t-test.

Fig. 3. The **s**ilencing of *MiSSP7.6* by RNAi impairs the establishment of ectomycorrhizal symbiosis with *P. tremula x alba*. A. Quantitative RT-PCR analysis of *MiSSP7.6* expression in *L. bicolor* lines expressing either the empty vector (CK-1, CK-2, CK-3) or *MiSSP7.6*-RNAi constructs (RNAi-1, RNAi-2, and RNAi-3). ECM, ectomycorrhizal roots. FLM, free-living mycelium. Error bars indicate ± standard deviation based on three technical replicates. Stars indicate that expression is statistically different from control

line 1 (CK1) in ECM using a t-test and a p-value cutoff < 0.05. B. Percentage of ectomycorrhizal root tips formed by strains expressing the empty vector (CK, control) or six independent *MiSSP7.6* RNAi lines, which were assayed three weeks after contact. Error bars represent ± standard deviation, n = 36. Stars indicate significant differences from six empty vector control using a t-test and a p-value cutoff < 0.05. C, D. Transverse sections of ectomycorrhizal root tips established by empty vector control transformant lines (**C**) or *MiSSP7.6*-RNAi lines (**D**). The *MiSSP7.6-RNAi* lines displayed a normal mantle sheath but less extensive Hartig net as compared to the empty vector control. Propidium iodide stains plant cell walls (in red) and wheat germ agglutinin alexa488 stains fungal cell walls (in green). fm, fungal mantle; ec, epidermal cell; cc, cortical cell; hn, Hartig net. Bars, 25 µm.

Fig. 4. Subcellular localization of MiSSP7.6 in *L. bicolor* hyphae. A. The green fluorescence was detected throughout hyphae cells for *MiSSP7.6*Δ*SP* fused to *EGFP* sequence. B. The green fluorescence accumulated in nuclei when EGFP sequence was fused to *L. bicolor* histone *H2B* gDNA. Green fluorescent and bright-field images were overlayed to produce the right panel images. Bars, 20 µm.

Fig. 5. MiSSP7.6 interacts with PtTrihelix1 and PtTrihelix2 transcription factors in yeast. The control colonies for the ProQuest Two-Hybrid System are shown in the upper part of the panel. Krev1/RalGDS-wt is a strong positive interacting pair, Krev1/RalGDS-m1 is a weak interacting pair, and Krev1/RalGDS-m2 is a pairing of proteins that show no detectable interaction. MiSSP7.6 without signal peptide (MiSSP7.6ΔSP) was used in the initial isolation of PtTrihelix1 and PtTrihelix2 from the YTH library screen. SC/-Leu-Trp

medium was used for mating selection. SC/-Leu-Trp-His+3AT and SC/-Leu-Trp-Ura medium were used for selection of interacting clones. The interaction strength was measured through the ß-Galactosidase activity on plates containing X-Gal (80 µg/mL). The false positive interactions were further eliminated by reverse YTH by 5FOA reverse selection. The combination of PtTrihelix1 and PtTrihelix2 with Krev1 was used as specific negative control.

Fig. 6. Interaction of the transcription factors PtTrihelix1 and PtTrihelix2 with MiSSP7.6, and homo-dimerization of PtTrihelix1 in plant nuclei. PtTrihelix1, PtTrihelix2, MiSSP7.6, and MiSSP7.6ΔSP (without signal peptide) were expressed in *N. benthamiana* epidermal leaves as recombinant proteins fused with the C-terminal SCFP and N-terminal Venus, respectively. A. and B. Co-expression of both of MiSSP7.6 and MiSSP7.6ΔSP with PtTrihelix1, resulted in strong fluorescence signals in the plant nuclei. C. Full-length MiSSP7.6 did not interact with PtTrihelix2, while D. MiSSP7.6 without signal peptide interacted with PtTrihelix2 in plant nuclei. E. Homo-dimerization of PtTrihelix1 in plant nuclei. F. No fluorescence signal was observed for the coexpression of PtTrihelix1 with PtTrihelix2. Fluorescence and bright-field images of the same leaf cells were superimposed to produce the overlaid images. Bars, 20 µm.

Fig. 7. PtTrihelix1, but not PtTrihelix2, altered the localization of MiSSP7.6 in tobacco cells. A. Co-expression of EGFP-MiSSP7.6 and RFP-PtTrihelix1 in *N. benthamiana* leaf cells. RFP-PtTrihelix1 (red) co-localized with EGFP-MiSSP7.6 (green) in the nuclei as shown by the yellow color in the overlaid images. B. The localization of the EGFP protein control did not change when co-expressed with RFP-PtTrihelix1. C. Co-expression of

EGFP-MiSSP7.6 and RFP-PtTrihelix2 and RFP-PtTrihelix2 caused co-localization of the fusion proteins in the nuclei, but did not affect cytoplasmic localization of EGFP-MiSSP7.6. Note that the cytoplasmic localization of EGFP-MiSSP7.6 was barely detectable when co-expressed with PtTrihelix1 (A). Fluorescent images of *N. benthamiana* epidermal leaf cells were taken by CLSM three-days post infiltration with *Agrobacterium* cells harboring appropriate constructs. Overlaid images were generated by overlaying green, red and bright-field images. N stands for nuclei. Scale bars represent 20 µm.

Supporting Information

Fig. S1 The predicted signal peptide of MiSSP7.6 protein as predicted by SignalP v4.1.

Fig. S2 Growth phenotypes of *MiSSP7.6* RNAi lines of *L. bicolor* S238N under axenic growth conditions.

Fig. S3 Subcellular localization of MiSSP7.6 in tobacco cells.

Fig. S4 Structural features of the PtTrihelix1 and PtTrihelix2 transcription factors.

Fig. 1.

A

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NAVSPPGQHCCGISPDVQYCLPIGTIC

B

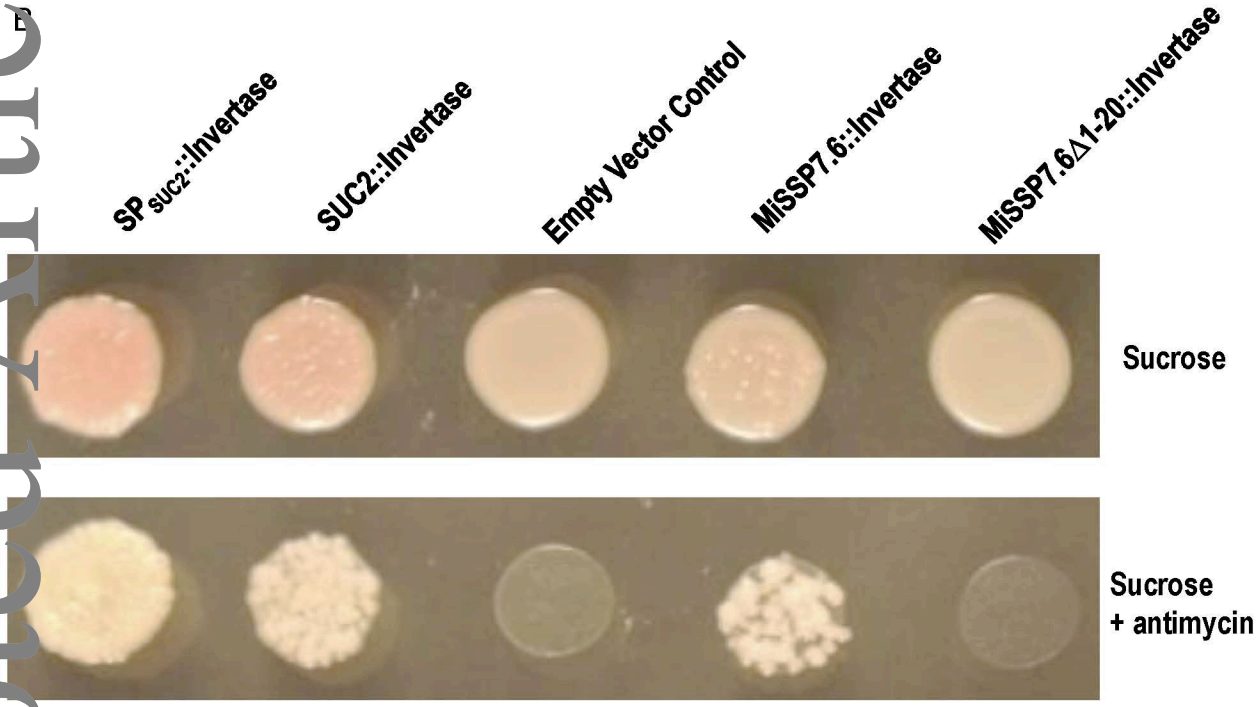

Fig. 2.

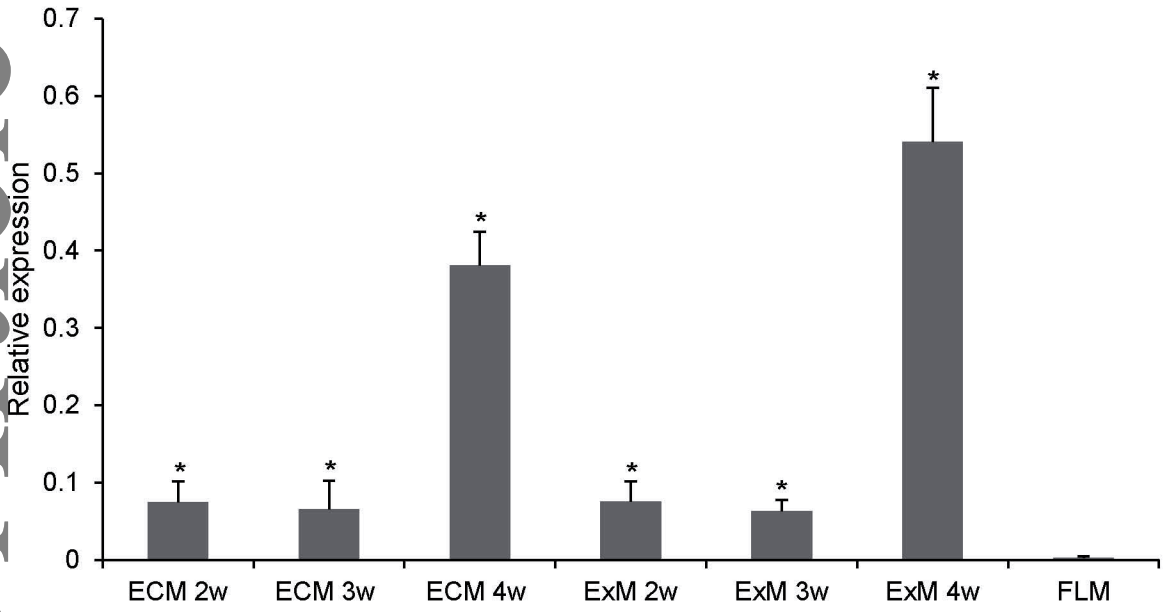

Fig. 3.

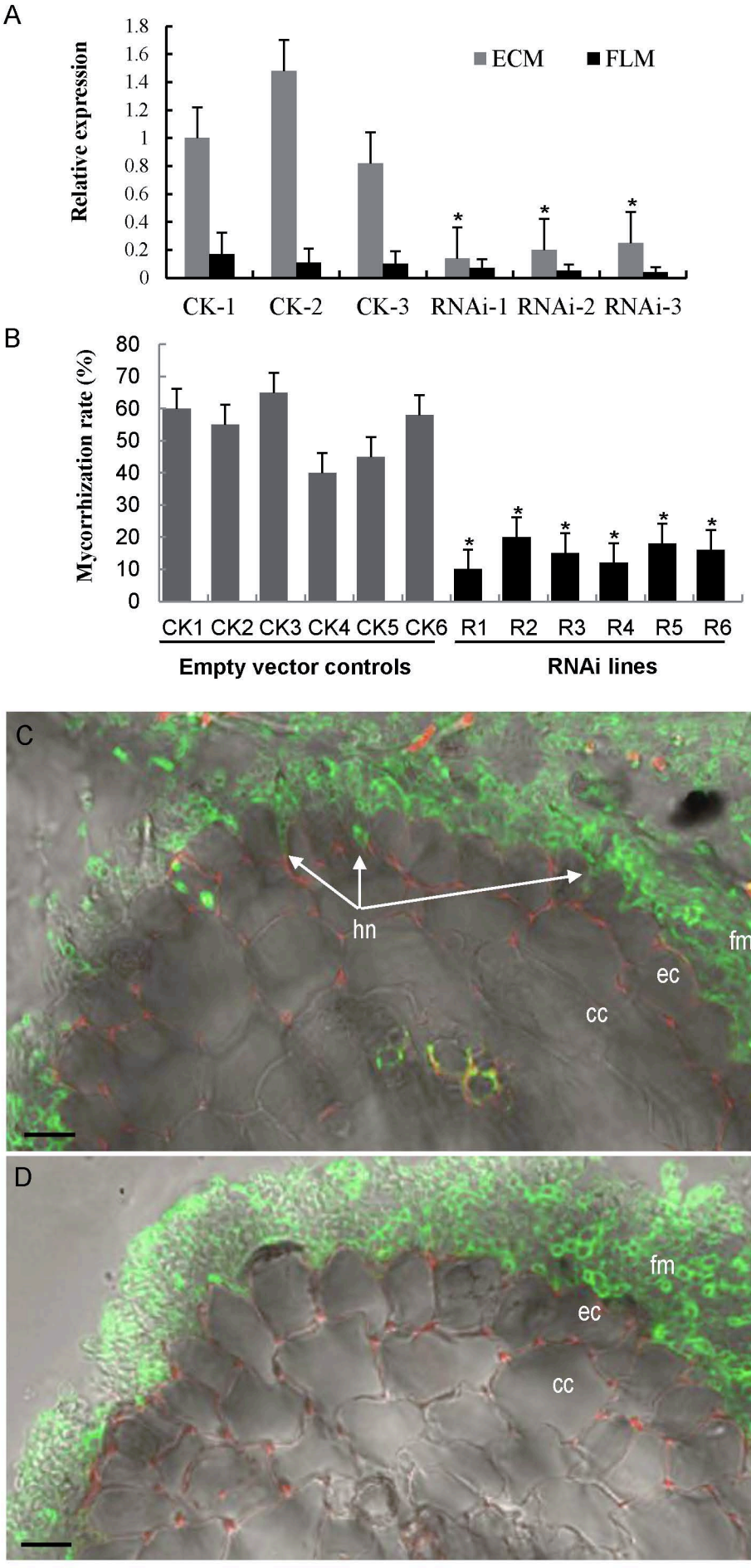

Fig. 4

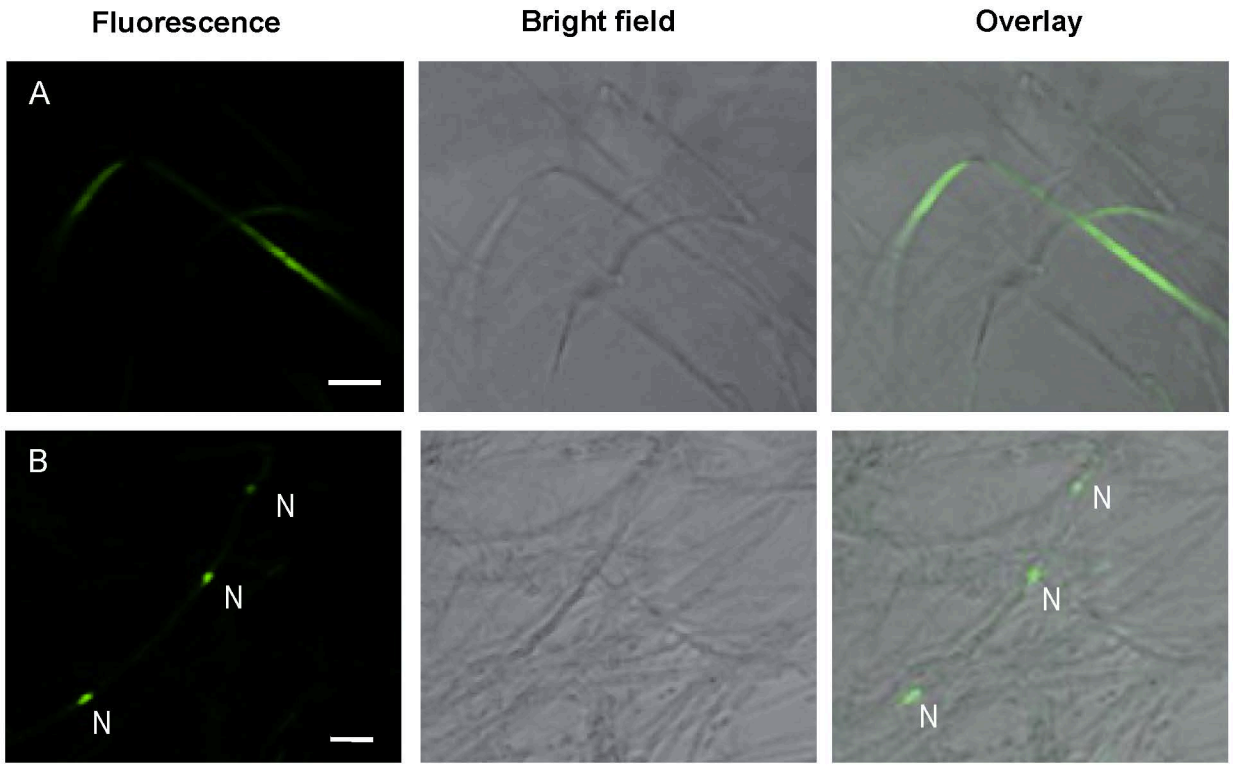

Fig. 5.

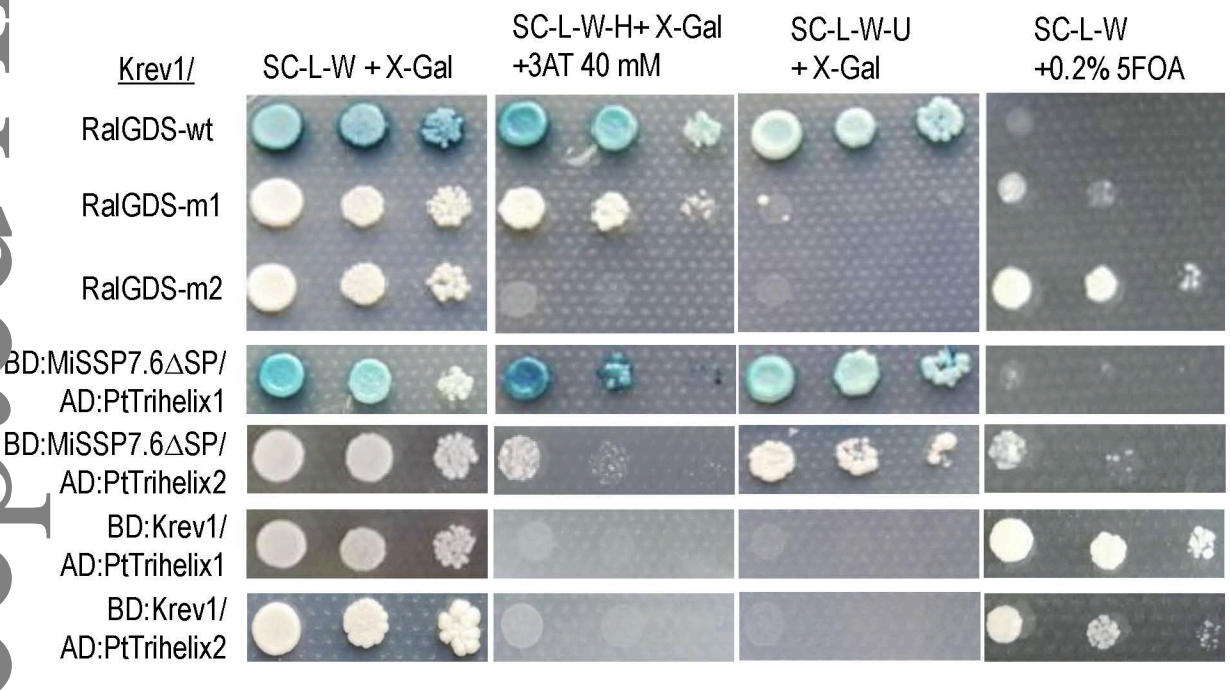

Fig. 6.

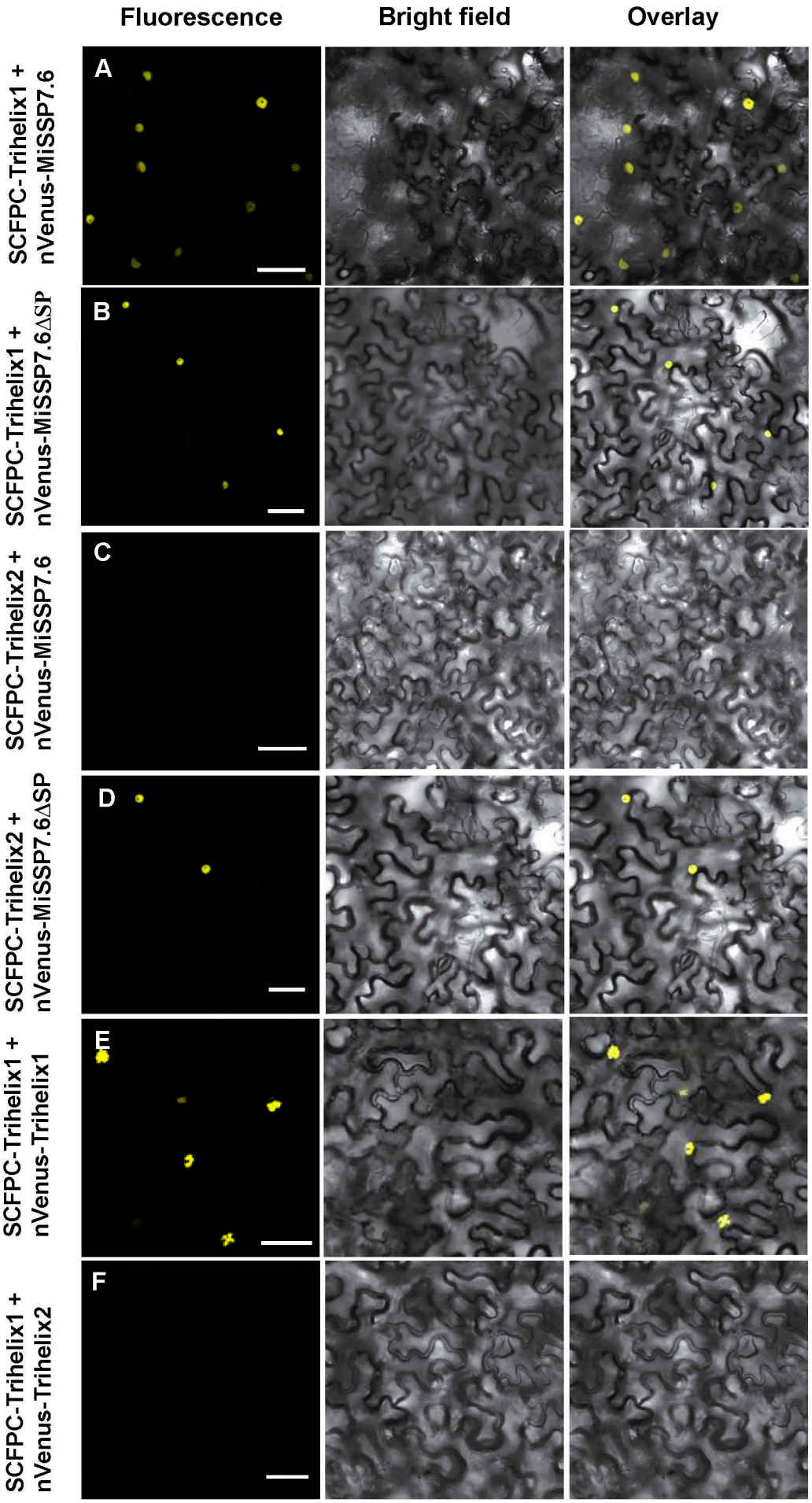

Fig. 7.