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Report

A Secreted Effector Protein of *Laccaria bicolor* Is Required for Symbiosis Development

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Summary

Soil-borne mutualistic fungi, such as the ectomycorrhizal fungi, have helped shape forest communities worldwide over the last 180 million years through a mutualistic relationship with tree roots in which the fungal partner provides a large array of nutrients to the plant host in return for photosynthetically derived sugars [1, 2]. This exchange is essential for continued growth and productivity of forest trees, especially in nutrient-poor soils. To date, the signals from the two partners that mediate this symbiosis have remained uncharacterized. Here we demonstrate that *MYCORRHIZAL iNDUCED SMALL SECRETED PROTEIN 7* (*MiSSP7*), the most highly symbiosis-upregulated gene from the ectomycorrhizal fungus *Laccaria bicolor* [3], encodes an effector protein indispensible for the establishment of mutualism. MiSSP7 is secreted by the fungus upon receipt of diffusible signals from plant roots, imported into the plant cell via phosphatidylinositol 3-phosphate-mediated endocytosis, and targeted to the plant nucleus where it alters the transcriptome of the plant cell. *L. bicolor* transformants with reduced expression of MiSSP7 do not enter into symbiosis with poplar roots. MiSSP7 resembles effectors of pathogenic fungi, nematodes, and bacteria that are similarly targeted to the plant nucleus to promote colonization of the plant tissues [4–9] and thus can be considered a mutualism effector.

Results

MiSSP7 Is Produced upon Receipt of Plant Root Secretions

MYCORRHIZAL iNDUCED SMALL SECRETED PROTEIN 7 (*MiSSP7*) encodes a mature peptide of 68 aa (Figure 1A) that lacks cysteine residues. It was previously found that *MiSSP7* mRNA and protein accumulate in mature mycorrhizal root tips [3]. To determine whether *MiSSP7* is expressed earlier in the colonization process, we performed transcriptomic measurements and protein immunolocalization throughout a 12-week time course of mycorrhization of *Populus trichocarpa* roots. *MiSSP7* transcripts were not detected in free-living mycelium but were detected during all stages of colonization (Figure 1B). Similarly, MiSSP7 protein was only detected in fungal hyphae colonizing roots (Figure 1C; see also Figures S1A–S1L available online). Because fungal colonies of *Laccaria bicolor* separated from direct contact with poplar roots by a cellophane membrane produced MiSSP7, direct contact with poplar roots was not necessary for MiSSP7 induction (Figures S1M–S1X). Activation of MiSSP7 was not ectomycorrhizal (ECM) host plant specific, because roots of the nonhost plant *Arabidopsis thaliana* could also induce its production (Figures S1W and S1X). Therefore, MiSSP7 is produced in *L. bicolor* upon receipt of diffusible signals from plant roots throughout the development of a mycorrhizal root tip.

MiSSP7 Is Imported into the Plant Cell

MiSSP7 was computationally predicted to be secreted into the plant apoplastic space [3]. Its localization after secretion was further investigated here. Using immunofluorometric labeling, we found that MiSSP7 enters plant cells and accumulates in the plant nuclei (Figure 1C). These results were corroborated by the nuclear localization of 5,6-carboxyfluorescein (FAM)-tagged MiSSP7 protein in poplar root and suspension-cultured cells (Figure 1D; Figures S2A–S2D). Transgenic poplar root cells expressing *MiSSP7* (Figures S2F and S2G) also accumulated MiSSP7 protein in their nuclei. Attachment of a nuclear export signal to the MiSSP7 peptide labeled with a FAM fluorochrome resulted in accumulation of the protein in the nuclear rim and reduced time in the plant nucleus (Figure 1D; Figure S2H). Access to the nucleus was not based on the small size of the MiSSP7 protein, because nuclear localization was significantly reduced in a truncated version of MiSSP7 despite cell entry (Figure 1D). Whereas no nuclear localization signal was identified for MiSSP7 using a bioinformatics approach, all truncations to MiSSP7 used in this study (Figure S3) that gained access to the cell exhibited a significantly reduced nuclear localization (Figure S3). Thus, the full protein, rather than one discrete nuclear localization motif, may be needed for nuclear import. Nuclear localization occurs quickly (Figure 1E), within 30 min of incubation. Only 86% of nuclei were labeled, presumably because cells in the culture with different developmental states differentially take up external proteins [10]. Upon removal of MiSSP7 from the medium, nuclear localization, as denoted by fluorescently labeled nuclei, decreased rapidly (Figure 1E). This quick turnover of nuclear MiSSP7 would be consistent with a potential role for MiSSP7 in signaling between *L. bicolor* and plant roots.

MiSSP7 Enters Plant Cells via Endocytosis

Exogenous proteins, including fungal effectors, have been reported to enter plant cells via one of two main pathways: lipid raft-mediated endocytosis [11] or macropinocytosis [10, 12]. Only inhibitors of the endocytosis pathway significantly inhibited the cell entry of MiSSP7 (Figure 2A; $p < 0.01$). Dead root cells did not take up MiSSP7. Additionally, treatment of root cells with brefeldin A (BFA), an inhibitor of endosome vesicular trafficking, concentrated MiSSP7 and FM4-64 (a fluorescent marker of plasma membranes and endocytotic bodies) into a previously described "BFA compartment" [13],

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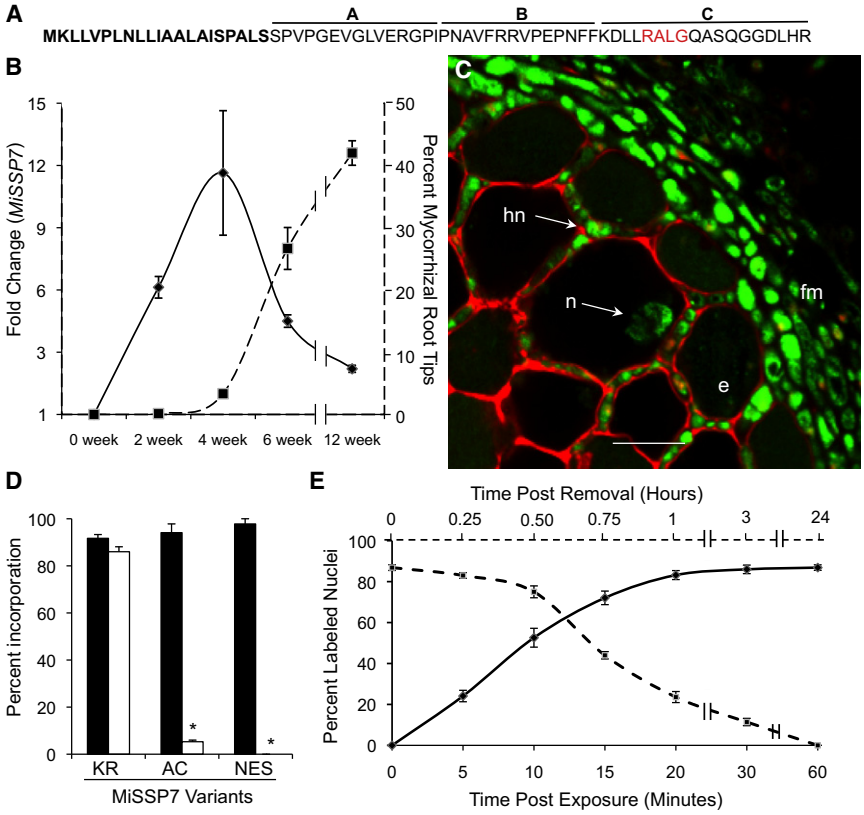

Figure 1. MiSSP7 Expression and Accumulation during Mycorrhizal Development

(A) Protein sequence of MiSSP7, with the predicted secretory leader in bold. Sequences used in truncation experiments are labeled A, B, and C above the corresponding amino acids. The identified motif required for plant cell entry (RALG) is indicated in red. For further information on constructs used in mutational and truncation experiments, please see Figure S3.

(B) Quantification of MiSSP7 transcripts during mycorrhization (solid line) versus percent mycorrhization (dashed line; n = 6).

(C) Indirect immunolocalization of MiSSP7 (green signal) in colonized root. The following abbreviations are used: hn, Hartig net; n, nucleus; e, root epidermal cell layer; fm, fungal mantle. Plant cell walls were stained with propidium iodide (red signal). Scale bar represents 20 μm.

(D) Cell entry (black bars) and nuclear localization (white bars) of MiSSP7 (KR), a truncated version of MiSSP7 (AC), and MiSSP7 with a nuclear exclusion signal attached (NES) (defined in Figure S3).

(E) Incorporation of MiSSP7 into the plant cell nucleus (solid line) and the rate of loss of MiSSP7 from the plant nucleus upon removal of MiSSP7 from the supernatant (dashed line).

All values are shown as mean ± standard error; n = 3 unless otherwise noted.

reinforcing the hypothesis that MiSSP7 actively enters the cell via endocytosis (Figures S2I and S2J). BFA treatment also blocked MiSSP7 localization to the nucleus (Figure 2B). Truncations of MiSSP7 were created to identify regions responsible for recognition and uptake into the plant cell (Figure S3). Only peptides containing the region from amino acids 51 to 58 could be imported into plant cells (Figure 2C). Just these eight amino acids were sufficient to initiate endocytosis of a fluorescent label into the plant root cell (Figure 2). Mutational analysis of this region within the context of the whole protein indicated that the amino acids RALG were necessary for entry into root cells (Figure 2C). Given the conformity of RALG to RXLR-like sequences that enable phosphatidylinositol 3-phosphate (PI-3-P)-mediated host cell entry by some pathogenic effector proteins [11, 14–16], we analyzed the ability of MiSSP7 to bind PI-3-P and the related molecules PI-4-P and PI-5-P. MiSSP7 bound with the highest affinity to PI-3-P and to a lesser extent to PI-4-P, an interaction that required an intact RALG motif (Figure 3). Consistent with these results, myo-inositol-1,4-diphosphate, a competitive inhibitor of PI-3-P binding, and wortmannin, which depletes cell surface PI-3-P [11], also inhibited uptake of MiSSP7 into poplar root cells (Figure 2A) and into the BFA compartment of poplar cells (Figure S2J). Similarly, 1,3-IP2 and PEPP1, a protein that competitively binds PI-3-P [11], also inhibited entry into root cells (Figure 2A). Stability of the mutated proteins was not compromised as compared to wild-type MiSSP7 protein (Figure 3C). Therefore, the lack of entry of mutated proteins is not due to increased degradation of the protein before cell entry. Together, these results reinforce the conclusion that MiSSP7 enters the plant cell via PI-3-P-mediated endocytosis and that the RALG domain is necessary for this import.

MiSSP7 Production Is Critical for the Formation of the Hartig Net

To test the functional role of MiSSP7 in the development of a mycorrhizal root tip, we generated ten independent transgenic lines of *L. bicolor* with lowered production of MiSSP7, as demonstrated by reduced expression of the gene and the protein, using RNA silencing (RNAi) (Figure 4A; Figure S4; Table S1) [17, 18]. Because homologous gene replacement is not available in *L. bicolor*, this technique could not be used to delete the *MiSSP7* gene to analyze its impact on the establishment of symbiosis. The ability to form ECM root tips in the *L. bicolor missp7* silenced lines dropped significantly, from about 40% to 0%–3% mycorrhizal root tips, depending on the silenced line (Figure 4A). Of the few mycorrhizal root tips induced by *L. bicolor* silenced lines, the vast majority had an atypical morphology (Figure S4A). Empty vector transformants of *L. bicolor* mycorrhized similarly to wild-type *L. bicolor* (Figure 4A). Loss of MiSSP7 production did not affect the growth rate of the free-living mycelium (Table S1) and thus is unlikely to account for the reduced ability of the silenced lines to enter into symbiosis with poplar roots. Cross-sections of root tips colonized by silenced lines revealed formation of a mantle, a shallow Hartig net, and little accumulation of MiSSP7 protein (Figures S4C and S4D). Therefore, MiSSP7 is likely one of the components necessary for growth of ECM fungal hyphae into the root apoplast. Because RNAi precludes the use of transgenic overexpression of *MiSSP7* (e.g., driven by 35S) as a means to complement the fungal mutant lines, two experimental strategies—biochemical complementation and heterologous expression of *MiSSP7* in planta—were used to determine whether reduced expression of *MiSSP7* alone was responsible for the inability of *L. bicolor missp7* silenced lines

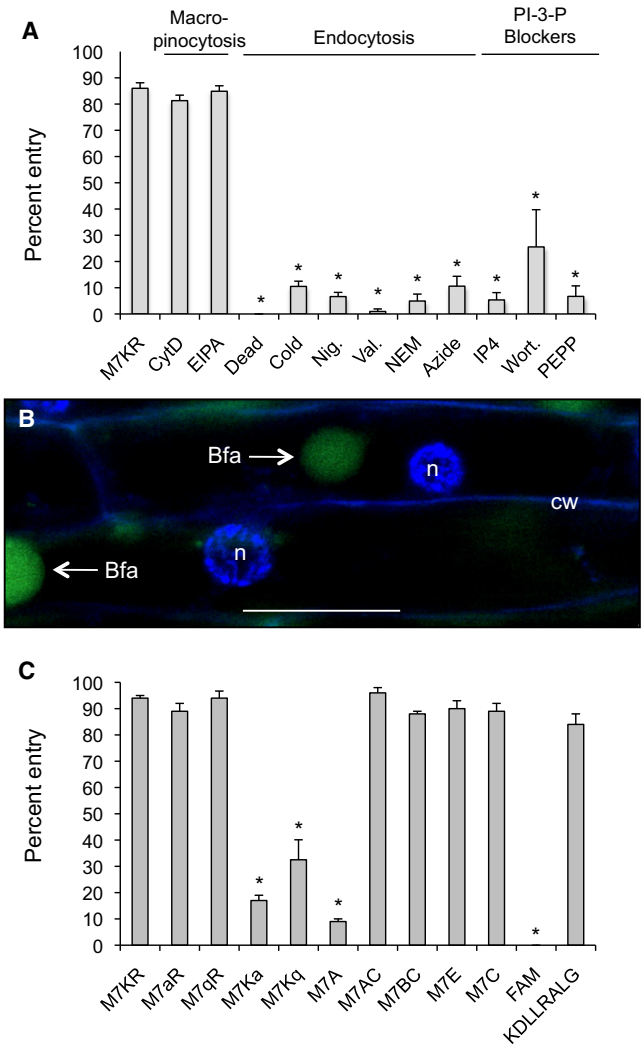

Figure 2. MiSSP7 Enters the Plant Cell via Endocytosis through Recognition of an RXLR-like Motif

(A) Cell entry of MiSSP7 (M7KR) incubated with the macropinocytosis inhibitors cytochalasin D (CytD) and 5-(N-ethyl-N-isopropyl)amiloride (EIPA); in dead roots (Dead); with the endocytosis inhibitors 4°C (Cold), nigericin (Nig.), valinomycin (Val.), N-ethylmaleimide (NEM), or sodium azide (Azide); or with the phosphatidylinositol 3-phosphate (PI-3-P) blockers myo-inositol-1,4-diphosphate (IP4, a PI-3-P analog), wortmannin (Wort., a phosphatidylinositol 3-kinase inhibitor), or PEPP1 (PEPP, a PI-3-P binding protein).

(B) Upon BFA incubation, fluorescently labeled MiSSP7 accumulates in the BFA compartment (green signal). The following abbreviations are used: Bfa, brefeldin A compartment; n, nucleus; cw, cell wall. Cell walls and nuclei were stained with DAPI (blue signal). Scale bar represents 40 μm.

(C) Cell entry of fluorescently labeled MiSSP7 (M7KR); MiSSP7 with mutated KDLL (M7aR, KDLL→AAAA; M7qR, KDLL→QDLL) or RALG motifs (M7Ka, RALG→AAAA; M7Kq, RALG→QALG); or MiSSP7 fragments containing region A only (M7A), A+C (M7AC), B+C (M7BC), half of A + C (M7E), or C only (M7C). See Figure 1A and Figure S3 for sequences. As compared to the FAM molecule alone (FAM), the KDLLRALG peptide without any other part of the MiSSP7 protein (KDLLRALG) is able to enter the cell via endocytosis.

All values are shown as mean ± standard error; n = 3. *$p < 0.01$ versus wild-type MiSSP7 entry.

to form a full intraradicular Hartig net. In both cases, replacement of MiSSP7 was able to complement the loss of MiSSP7 and reestablish hyphal penetration within the root (Figure 4B). Cell entry and nuclear localization were necessary for this complementation, because MiSSP7 peptides used in biochemical complementation that were mutated to be excluded from either the cell (MiSSP7Ka) or the nucleus (MiSSP7-NES) did not significantly affect the ingrowth of fungal hyphae (Figure 4B; Figure S3).

MiSSP7 Alters the Plant Cell Transcriptome

Because nuclear localization of MiSSP7 is necessary for promotion of root penetration by *L. bicolor*, it seemed likely that MiSSP7 could reprogram the transcriptome of the plant cell. To investigate which genes might be affected by the presence of MiSSP7, we analyzed the transcriptome of poplar roots incubated with MiSSP7 protein for 1 hr. Two hundred and twenty-five transcripts were significantly modulated (>2.5-fold; $p < 0.05$; Table S2; Table S3). A large portion of the genes most highly modulated by MiSSP7 are involved in alteration of the root architecture. Of the most highly modulated genes, transcripts from auxin-responsive genes like the auxin/indole-3-acetic acid (Aux/IAA) genes, *GH3*, and the small auxin-up RNA (SAUR) gene families were upregulated. In concert with genes implicated in alteration of the root architecture, among the downregulated transcripts is *CLAVATA3/ESR-RELATED 5* (*CLE5*). Because overexpression of CLE results in inhibited root growth [19], downregulation of *CLE5* by MiSSP7 might induce root growth. Furthermore, transcripts of genes implicated in cell wall remodeling (e.g., beta-glucosidase, pectinase, and extensin) and reactive oxygen species production (*GRIM REAPER*; [20]) were more abundant in MiSSP7-treated roots. To ensure that these effects on the transcriptome were due to MiSSP7 in the nucleus, and not due to a peptide effect, we analyzed the expression of a number of these genes in the presence of a mutant version of MiSSP7 that cannot enter the plant cell or nucleus (Table S3). None of the genes tested were significantly regulated by the mutant version of MiSSP7, indicating that the presence of MiSSP7 in the plant nucleus is indeed needed for alteration of the plant transcriptome in the manner demonstrated here. These results are interesting in light of the phenotype of *L. bicolor missp7* silenced lines, which are unable to penetrate between the cells of the root. Perhaps during root colonization, MiSSP7 may affect the maintenance and structure of the plant cell walls, or of the root architecture in general by affecting plant hormone signaling, to facilitate hyphal penetration between cells and establishment of the Hartig net.

Discussion

In order to establish MiSSP7 as a genuine mutualism effector that controls the establishment and/or maintenance of the symbiotic relationship, it was necessary to prove that (1) it is induced by the presence of a plant root, (2) it alters functioning of the plant cell, and (3) it is necessary for mycorrhizal symbiosis. Our results demonstrate that MiSSP7, upon secretion, is able to traverse the plant cell wall and membrane to localize to the nucleus and that this localization alters the transcriptional status of host trees. The results suggest that MiSSP7 may be considered a master mutualism effector involved in the reprogramming of plant cells to favor mutualism. In mutualistic bacteria, small numbers of master regulators also appear to mediate symbiosis [21], and the alteration of one gene can render a nonsymbiotic bacterium mutualistic [22]. The severity of the impact that loss of MiSSP7 has on the

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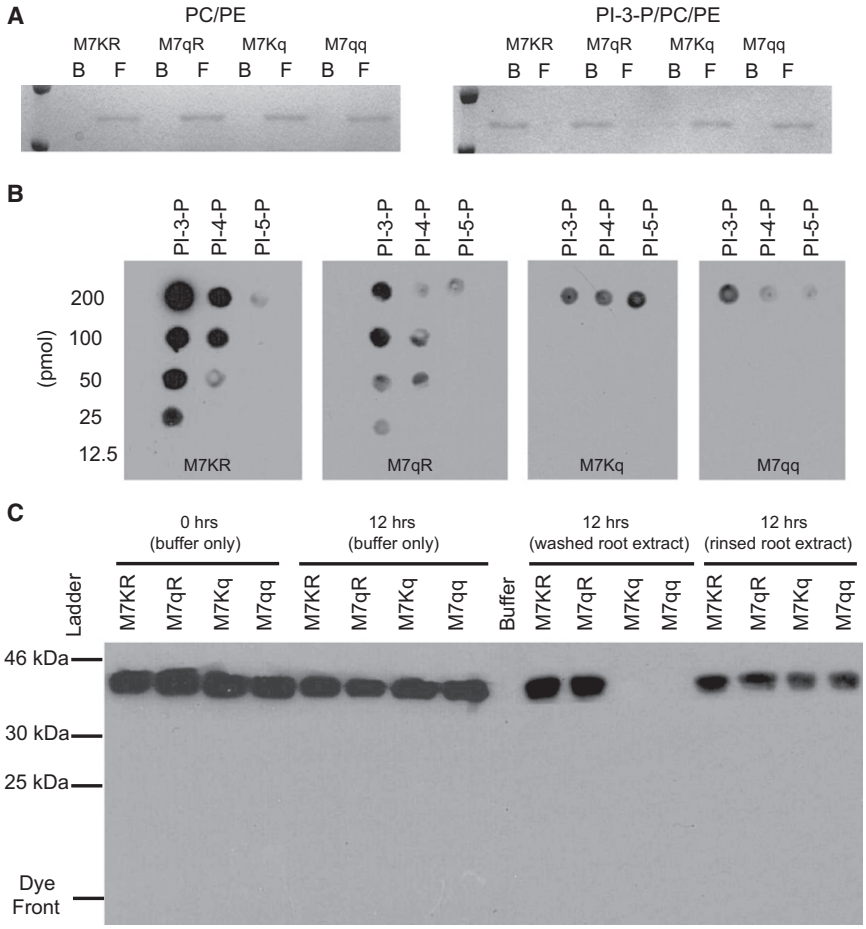

Figure 3. MiSSP7 Binding to Phosphoinositides Requires the Motif RALG

(A) Liposome-binding assays of wild-type and mutant MiSSP7 proteins to phosphatidylcholine and phosphatidylethanolamine alone (PC/PE) or to PC and PE with the addition of 10% PI-3-P (PI-3-P/PC/PE). "B" and "F" indicate liposome-bound and liposome-free proteins, respectively.

(B) Filter-binding assays of wild-type (M7KR) and mutant (M7qR, qDLLRALG; M7Kq, KDLLqALG; M7qq, qDLLqALG) MiSSP7 proteins to phosphoinositides. Binding only to the 200 pmol spot is too weak to enable cell entry [11].

(C) Stability and internalization assay of wild-type MiSSP7-GFP fusion protein and mutants in 25 mM MES buffer (pH 6.8; final concentration 1.0 mg/ml) after a 12 hr incubation with or without the presence of roots at 28°C or from root cell extracts. Western blotting shows no degredation products or smearing of either mutated or wild-type proteins under these conditions from buffer samples or cell extracts. Cell extracts demonstrate that only MiSSP7KR and MiSSP7qR are recovered from within the cell, and not MiSSP7Kq or MiSSP7qq, despite their presence in the buffer, reinforcing the finding that the RALG motif is necessary for cell entry.

establishment of mutualism by *L. bicolor* mutants supports its classification as a master regulator of symbiosis.

The data presented here for MiSSP7 shed an interesting light on how close symbiotic signals may be between mutualistic and pathogenic fungi. The current understanding of the evolution of symbiotic fungal lineages is that they derived from saprotrophic ancestors (e.g., brown rot fungi) and, through convergent evolution, a number of different phylogenies developed the ability to establish symbioses with plants [1]. Whereas dozens of effectors released by pathogenic fungi and oomycetes have been characterized, similar secreted effector-like proteins used to colonize host roots have not been characterized in a mutualistic system to date. Here we have shown that the most highly regulated *L. bicolor* transcript in symbiotic tissues, MiSSP7, is an effector-like protein necessary for the penetration of fungal hyphae into plant tissues and for the maintenance of symbiosis in the plant apoplast. This would mean that the steps leading to the colonization of roots by ECM symbionts, including the use of effectors to subjugate plant defenses or alter plant cell wall characteristics, may be closer in mechanism to their pathogenic cousins than originally thought. This idea is further reinforced by the finding that MiSSP7 binds to membrane phospholipids and enters the cell via endocytosis in a manner similar to pathogenic effectors containing RXLR and RXLR-like motifs [11, 14–16, 23]. Furthermore, the demonstration here of a mutualistic effector protein that reprograms the host transcriptome mirrors the role of fungal pathogen effectors [7, 9, 24, 25]. The symbiont *Glomus intraradices* also secretes a protein that interacts with the pathogenesis-related transcription factor ERF19 in the plant nucleus, contributing to the biotrophic development of arbuscular mycorrhizal fungi in roots by counteracting the plant immune program (see Kloppholz et al. [26] in this issue of *Current Biology*). This calls into question the very nature of the mutualistic relationship; perhaps it is very similar to some pathogenic relationships. Upon the release in 2006 of the genome for the fungal pathogen *Ustilago maydis*, which bears some hallmarks of the *L. bicolor* genome (reduced number of CAZymes, large number of effector-like SSPs), it was postulated that mutualistic fungi might use pathways similar to fungal pathogens to live in "pretend harmony" through the use of secreted effector proteins [27]. Our results here would indicate that this prediction is true. Given the key results obtained for MiSSP7, the role played by other ectomycorrhiza-upregulated small secreted proteins of *L. bicolor* [3] should be elucidated, as well as the identity of plant-based signals that may control *L. bicolor* growth within the root space.

Experimental Procedures

Plant and Fungal Material Used

Poplar lines *Populus trichocarpa* clone 101-74 and *P. tremula* × *P. alba* clone 717-1B4 were used. All mycorrhization trials (greenhouse and in vitro) used the *Laccaria bicolor* isolate S238N, the parental strain to the homokaryon used to sequence the genome [3].

Transformation of *L. bicolor* and Poplar

Transformation of *L. bicolor* S238N was performed using the RNAi/*Agrobacterium*-mediated transformation (AMT) vector for intron hairpin RNA (ihpRNA) expression, and transformation of *L. bicolor* vegetative mycelium used the pHg/pSILBA$_\gamma$ vector system as described in [18] using the full-length cDNA sequence of *MiSSP7*. Ten pHg/pS$_\gamma$MiSSP7 *L. bicolor* transformant strains were used in this study and were characterized as

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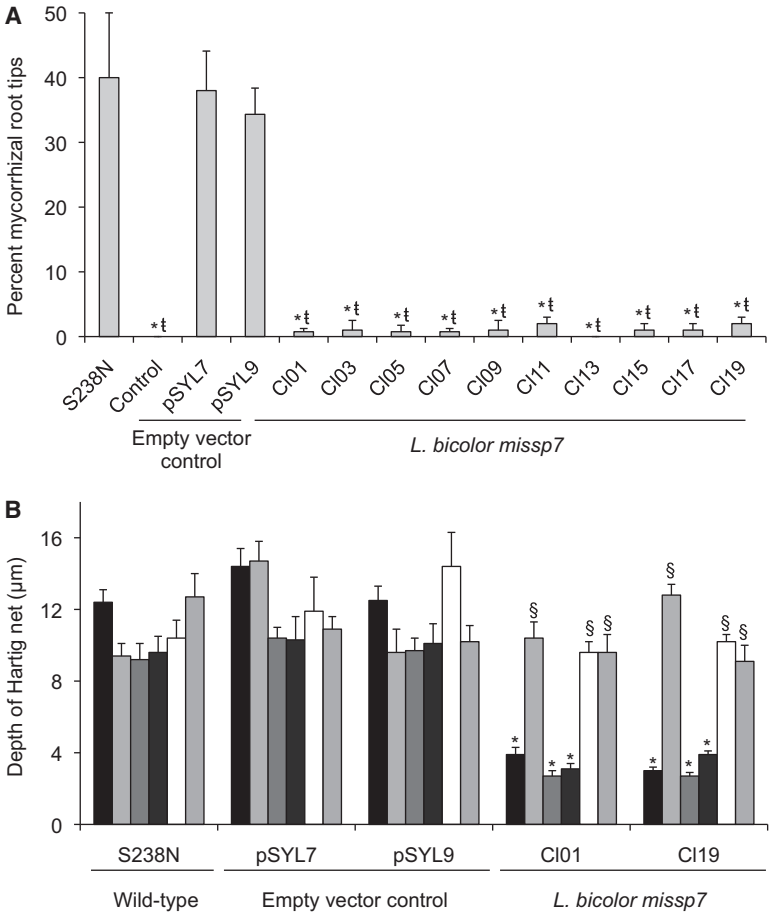

Figure 4. RNA Silencing of the *MiSSP7* Gene Significantly Reduces the Ability of *L. bicolor* to Form Mycorrhizal Root Tips and Form a Hartig Net

(A) Percentage of mycorrhizal root tips formed by wild-type *L. bicolor* S238N and two empty vector transformation controls (pSYL7 and 9) versus ten independent *L. bicolor missp7* RNAi strains (Cl01–Cl19; n = 10).

(B) Functional complementation of silenced lines measured by Hartig net depth. Cl01 and Cl19 are two *L. bicolor missp7* silenced lines; S238N, pSYL7, and pSYL9 are controls. Bars show the average of the Hartig net depth in mycorrhizal root tips from 2-week-old in vitro cultures treated with control solution lacking protein (black), followed in order by treatment with exogenous wild-type MiSSP7 protein (MiSSP7KR) (light gray), mutated MiSSP7 that cannot enter the cell (MiSSP7Ka) (medium gray), and tagged MiSSP7 that cannot enter the nucleus (MiSSP7KR-NES) (dark gray). The last two bars of each data set are each fungal strain in contact with two independent transgenic poplar lines expressing MiSSP7 (white and light gray).

All values are shown as mean ± standard error; n = 3 unless otherwise noted. *p < 0.01 versus *L. bicolor* S238N mycorrhization potential (A) or versus *L. bicolor* S238N Hartig net depth (B); ‡p < 0.01 versus mycorrhization potential from empty vector controls (pSYL7 and 9); §p < 0.01 versus untreated Hartig net depth for the respective fungal strain.

described in [17, 28]. Transformation of *P. tremula* × *P. alba* with *MiSSP7* lacking the secretion signal (primers listed in Supplemental Experimental Procedures) was performed as described in [29] using the pORE-E2 vector under the constitutive HPL promoter [30].

Poplar Mycorrhization Experiments

Fungal cultures and mycorrhization experiments of *P. trichocarpa* (greenhouse experiments) or of *P. tremula* × *P. alba* clone 717-1B4 (in vitro experiments) by *L. bicolor* and *L. bicolor missp7* transgenic lines were performed as previously described for *P. trichocarpa* [3] and for *P. tremula* × *P. alba* as previously described in [31]. At least three biological replicates per time point were analyzed for mycorrhization potential or 10–20 biological replicates for the percent of colonized roots for each of the ten *L. bicolor missp7* mutant lines (Cl01, Cl03, Cl05, Cl07, Cl09, Cl11, Cl13, Cl15, Cl17, and Cl19). Two independent empty vector transformant *L. bicolor* lines (pSYL7 and pSYL9) were also tested for their ability to colonize roots (in vitro).

Complementation of Two *L. bicolor missp7* Silenced Lines

Using the in vitro assay [31], either wild-type *P. tremula* × *P. alba* clone 717-1B4 or one of two mutant 717-1B4 lines overexpressing *MiSSP7* were placed in direct contact with *L. bicolor missp7* line Cl01 or Cl19 (chosen because of intergenic T-DNA insertions), *L. bicolor* S238N, or *L. bicolor* pSYL7 or pSYL9 (empty vector-transformed lines) as controls. After fungal colonization, either 5 μl of 3.4 μM MiSSP7 (or MiSSP7Ka or MiSSP7-NES) in liquid Murashige and Skoog (MS) medium or a control solution of liquid MS medium was applied to newly emerged lateral roots every 2 days for a one-week period. Transgenic poplar roots expressing *MiSSP7* were not dosed with peptide but were left in contact with either the transgenic fungus or wild-type *L. bicolor* for two weeks as described in [31]. Sections of treated roots were stained with 1% UVtex to visualize the development of the Hartig net. All microscopy was performed using a Bio-Rad Radiance 2100 AGR3Q-BLD Rainbow microscope. The depth of hyphal penetration into the host root was measured using ImageJ, with a minimum of three measurements each from root from three biological replicates.

Microscopy and Immunolocalization

Immunolocalization of MiSSP7 was performed as described in [3], and plant cell walls were stained with propidium iodide.

Peptide Application Experiments

Poplar roots and poplar suspension cells were exposed to a fluorescently tagged synthetic version of the MiSSP7 protein (with or without different mutations or truncations; Figure S3) produced by Pi Proteomics (Huntsville, Alabama). Purity of all peptides used was verified by analytical high-performance liquid chromatography. Young roots of live 717-1B4 plants or 717-1B4 suspension cells [grown in the absence of light in liquid MS medium supplemented with 5 mM 6-(γ,γ-dimethylallylamino) purine and 100 nM 1-naphthaleneacetic acid] were acclimatized to new (hormoneless) MS medium for 16 hr prior to the addition of MiSSP7 peptide (or its mutant counterparts) to a final concentration of 3.4 μM. Rooted plants were incubated in this solution for 2 hr in the light at 24°C, whereas suspension cells were incubated for 30 min in the dark with shaking. Plant nuclei were stained with DAPI for 20 min. Macropinocytosis and endocytosis experiments were performed as described in [10–12]. For brefeldin A experiments, 717-1B4 roots were preincubated in liquid MS medium dosed with 10^{-4} M brefeldin A (Sigma-Aldrich) as in [32] for 30 min, after which MiSSP7 was added and left for an additional 1.5 hr. For the final 10 min of incubation, the fluorescent marker of endocytosis FM4-64 (Invitrogen) was added. Live roots were visualized immediately (minimum of three independent biological replicates per experiment, with a minimum of 30 cells counted per biological replicate).

PI-3-P Binding and Cell Entry

MiSSP7-GFP fusion proteins were produced in and purified from *E. coli* (BL21DE3). Cell entry of MiSSP7 fusions into root cells was performed as described in [11]. Binding of MiSSP7 to liposomes or phospholipids PI-3-P, PI-4-P, and PI-5-P and western blotting were performed as described in [11]. Note that for filter phospholipid binding assays, purified, defatted bovine serum albumin (Sigma-Aldrich) must be used as the blocking agent [11]; other blocking agents, particularly milk, may interfere with phosphoinositide binding.

RNA Extraction, cDNA Synthesis, Microarray, and Quantitative qPCR

Relative quantification of *MiSSP7* transcripts during fungal colonization of *P. trichocarpa* lateral roots was performed using free-living mycelium as a control. Synthesis of cDNA from total RNA was performed using the iScript kit (Bio-Rad) for qPCR procedures or the SMART PCR cDNA Synthesis kit (Clontech) according to the manufacturer's instructions for microarray analysis. Microarray experiments were performed as described in [3].

A Student's t test with Benjamini-Hochberg false discovery rate multiple testing correction was applied to the data using ArrayStar software (DNASTAR). Transcripts with a significant p value (<0.05) and $\geq$2.5-fold change in transcript level were considered as differentially expressed.

To verify the results of the microarray experiments, we analyzed by quantitative PCR ten of the genes most regulated by MiSSP7. Fold changes in gene expression were based on $\Delta\Delta$Ct calculations [33] and are reported in Table S3.

Statistical Analyses

At least three independent biological replicates were performed for each test outlined in this study to ensure reproducibility and significance of data reported. A Student's two-tailed independent t test was used to determine the significance ($p < 0.01$) of all results except microarray data, for which $p < 0.05$ was used.

For more detailed methods, please refer to Supplemental Experimental Procedures.

Accession Numbers

The complete microarray expression data set has been deposited at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the series accession number GSE29050.

Supplemental Information

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.05.033.

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References

1. Hibbett, D.S., and Matheny, P.B. (2009). The relative ages of ectomycorrhizal mushrooms and their plant hosts estimated using Bayesian relaxed molecular clock analyses. BMC Biol. *7*, 13.
2. Martin, F., Kohler, A., Murat, C., Balestrini, R., Coutinho, P.M., Jaillon, O., Montanini, B., Morin, E., Noel, B., Percudani, R., et al. (2010). Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature *464*, 1033–1038.
3. Martin, F., Aerts, A., Ahrén, D., Brun, A., Danchin, E.G.J., Duchaussoy, F., Gibon, J., Kohler, A., Lindquist, E., Pereda, V., et al. (2008). The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature *452*, 88–92.
4. Marti, M., Good, R.T., Rug, M., Knuepfer, E., and Cowman, A.F. (2004). Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science *306*, 1930–1933.
5. Gu, K., Yang, B., Tian, D., Wu, L., Wang, D., Sreekala, C., Yang, F., Chu, Z., Wang, G.-L., White, F.F., and Yin, Z. (2005). R gene expression induced by a type-III effector triggers disease resistance in rice. Nature *435*, 1122–1125.
6. He, P., Shan, L., Lin, N.C., Martin, G.B., Kemmerling, B., Nürnberger, T., and Sheen, J. (2006). Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in *Arabidopsis* innate immunity. Cell *125*, 563–575.
7. Huang, G., Dong, R., Allen, R., Davis, E.L., Baum, T.J., and Hussey, R.S. (2006). A root-knot nematode secretory peptide functions as a ligand for a plant transcription factor. Mol. Plant Microbe Interact. *19*, 463–470.
8. Lee, S.-W., Han, S.W., Sririyanum, M., Park, C.J., Seo, Y.S., and Ronald, P.C. (2009). A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. Science *326*, 850–853.
9. Doehlemann, G., van der Linde, K., Assmann, D., Schwammbach, D., Hof, A., Mohanty, A., Jackson, D., and Kahmann, R. (2009). *Pep1,* a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. PLoS Pathog. *5*, e1000290.
10. Chang, M., Chou, J.C., and Lee, H.J. (2005). Cellular internalization of fluorescent proteins via arginine-rich intracellular delivery peptide in plant cells. Plant Cell Physiol. *46*, 482–488.
11. Kale, S.D., Gu, B., Capelluto, D.G., Dou, D., Feldman, E., Rumore, A., Arredondo, F.D., Hanlon, R., Fudal, I., Rouxel, T., et al. (2010). External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell *142*, 284–295.
12. Chang, M., Chou, J.C., Chen, C.P., Liu, B.R., and Lee, H.J. (2007). Noncovalent protein transduction in plant cells by macropinocytosis. New Phytol. *174*, 46–56.
13. Satiat-Jeunemaitre, B., Cole, L., Bourett, T., Howard, R., and Hawes, C. (1996). Brefeldin A effects in plant and fungal cells: something new about vesicle trafficking? J. Microsc. *181*, 162–177.
14. Dou, D., Kale, S.D., Wang, X., Jiang, R.H., Bruce, N.A., Arredondo, F.D., Zhang, X., and Tyler, B.M. (2008). RXLR-mediated entry of *Phytophthora sojae* effector *Avr1b* into soybean cells does not require pathogen-encoded machinery. Plant Cell *20*, 1930–1947.
15. van West, P., de Bruijn, I., Minor, K.L., Phillips, A.J., Robertson, E.J., Wawra, S., Bain, J., Anderson, V.L., and Secombes, C.J. (2010). The putative RxLR effector protein SpHtp1 from the fish pathogenic oomycete *Saprolegnia parasitica* is translocated into fish cells. FEMS Microbiol. Lett. *310*, 127–137.
16. Rafiqi, M., Gan, P.H., Ravensdale, M., Lawrence, G.J., Ellis, J.G., Jones, D.A., Hardham, A.R., and Dodds, P.N. (2010). Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. Plant Cell *22*, 2017–2032.
17. Kemppainen, M.J., Duplessis, S., Martin, F., and Pardo, A.G. (2009). RNA silencing in the model mycorrhizal fungus *Laccaria bicolor*: gene knock-down of nitrate reductase results in inhibition of symbiosis with *Populus*. Environ. Microbiol. *11*, 1878–1896.
18. Kemppainen, M.J., and Pardo, A.G. (2010). pHg/pSILBAγ vector system for efficient gene silencing in homobassidiomycetes: optimization of ihpRNA - triggering in the mycorrhizal fungus *Laccaria bicolor*. Microb. Biotechnol. *3*, 178–200.
19. Miwa, H., Kinoshita, A., Fukuda, H., and Sawa, S. (2009). Plant meristems: CLAVATA3/ESR-related signaling in the shoot apical meristem and the root apical meristem. J. Plant Res. *122*, 31–39.
20. Wrzaczek, M., Brosché, M., Kollist, H., and Kangasjärvi, J. (2009). Arabidopsis GRI is involved in the regulation of cell death induced by extracellular ROS. Proc. Natl. Acad. Sci. USA *106*, 5412–5417.
21. Ferenci, T. (2008). The spread of a beneficial mutation in experimental bacterial populations: the influence of the environment and genotype on the fixation of rpoS mutations. Heredity *100*, 446–452.
22. Marchetti, M., Capela, D., Glew, M., Cruveiller, S., Chane-Woon-Ming, B., Gris, C., Timmers, T., Poinsot, V., Gilbert, L.B., Heeb, P., et al. (2010). Experimental evolution of a plant pathogen into a legume symbiont. PLoS Biol. *8*, e1000280.
23. Whisson, S.C., Boevink, P.C., Moleleki, L., Avrova, A.O., Morales, J.G., Gilroy, E.M., Armstrong, M.R., Grouffaud, S., van West, P., Chapman, S., et al. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature *450*, 115–118.
24. Shan, W., Cao, M., Leung, D., and Tyler, B.M. (2004). The Avr1b locus of *Phytophthora sojae* encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene Rps1b. Mol. Plant Microbe Interact. *17*, 394–403.
25. Wang, X.H., Mitchum, M.G., Gao, B., Li, C., Diab, H., Baum, T.J., Hussey, R.S., and Davis, E.L. (2005). A parasitism gene from a plant-parasitic nematode with function similar to CLAVATA3/ESR (CLE) of *Arabidopsis thaliana*. Mol. Plant Pathol. *6*, 187–191.
26. Kloppholz, S., Kuhn, H., and Requena, N. (2011). A secreted fungal effector of *Glomus intraradices* promotes symbiotic biotrophy. Curr. Biol. *21*, in press. Published online July 14, 2011. 10.1016/j.cub.2011.06.044.

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27. Kämper, J., Kahmann, R., Bölker, M., Ma, L.J., Brefort, T., Saville, B.J., Banuett, F., Kronstad, J.W., Gold, S.E., Müller, O., et al. (2006). Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. Nature *444*, 97–101.
28. Kemppainen, M.J., Duplessis, S., Martin, F., and Pardo, A.G. (2008). T-DNA insertion, plasmid rescue and integration analysis in the model mycorrhizal fungus *Laccaria bicolor*. Microb. Biotechnol. *1*, 258–269.
29. Harrison, E., Bush, M., Plett, J.M., McPhee, D.P., Vitez, R., O'Malley, B., Sharma, V., Bosnich, W., Séguin, A., MacKay, J., and Regan, S. (2007). Diverse developmental mutants revealed in an activation-tagged population of poplar. Can. J. Bot. *85*, 1071–1081.
30. Coutu, C., Brandle, J., Brown, D., Brown, K., Miki, B., Simmonds, J., and Hegedus, D.D. (2007). pORE: a modular binary vector series suited for both monocot and dicot plant transformation. Transgenic Res. *16*, 771–781.
31. Felten, J., Kohler, A., Morin, E., Bhalerao, R.P., Palme, K., Martin, F., Ditengou, F.A., and Legué, V. (2009). The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and Arabidopsis through auxin transport and signaling. Plant Physiol. *151*, 1991–2005.
32. Baluska, F., Hlavacka, A., Samaj, J., Palme, K., Robinson, D.G., Matoh, T., McCurdy, D.W., Menzel, D., and Volkmann, D. (2002). F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. Plant Physiol. *130*, 422–431.
33. Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45.