New Phytologist Supporting Information

Article title: VAPYRIN attenuates defence by repressing PR gene induction and localised lignin accumulation during arbuscular mycorrhizal symbiosis of Petunia hybrida

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Notes S1 - Supplementary Results

Medicago truncatula sym mutants do not accumulate lignin

In order to assess whether the lignin accumulation phenotype in petunia vpy mutants is related to a conserved function of VPY in suppression of defense, we tested whether *M. truncatula* vpy mutants showed a similar response as petunia vpy mutants. Furthermore, we included a number of additional *M. truncatula* mutants with a defect in AM symbiosis (Table S6), namely dmi2-1 (defective in SYMBIOSIS RECEPTOR-LIKE KINASE, SYMRK) (Endre et al., 2002), dmi3-1 (defective in CALCIUM AND CALMODULIN-DEPENDENT PROTEIN KINASE, CaMK) (Lévy et al., 2004; Mitra et al., 2004), raml-1 (defective in the RAM1 orthologue of petunia RAM1) (Gobbato et al., 2012), and nsp2-2 (defective in NODULATION SIGNALLING PATHWAY2, NSP2) (Kalo et al., 2005). As in the case of petunia, the mutants (and the wild type) were inoculated by chive nurse plants to achieve comparable levels of overall root colonization (>50% root length colonization) in all Medicago genotypes. Phenotypic assessment of these essentially confirmed all the symbiotic defects described previously (Fig. S9, S10). While the wild-type accessions A17 and R108 sustained the formation of hyphal coils in external cells (Fig. S9a,b), and of arbuscules in the root cortex (Fig. S9c), fungal penetration of the root surface was inhibited in dmi2 and dmi3 mutants (Fig. S9d,f). However, due to the strong inoculum potential of nurse plants, the fungus eventually entered the roots of both mutant genotypes. Subsequent cortical colonization was, however, markedly different: In dmi3-1, only intercellular hyphal colonization was observed (Fig. S9e), while in dmi2-1, arbuscules were formed at high frequency (Fig. S9g), as previously described (Demchenko et al., 2004).

Raml-1 and nsp2-2 showed milder phenotypes (Fig. S10a-d). In both cases, hyphal coils were formed upon penetration of the root surface (Fig. S10a,c), however, arbuscule formation was affected in contrasting ways: raml-1 mutants only allowed for the formation of small ill-defined arbuscular structures (Fig. S10b, arrows), as described before (Park et al., 2015; Rich et al., 2015). In contrast, nsp2-2 contained patchy colonization with apparently normal arbuscules interspersed with arbuscule-free regions or retarded arbuscules (Fig. S10d, arrows), implicating NSP2 in both, nodulation as well as AM (Maillet et al., 2011).

Of particular interest was the *Medicago* vpy-2 mutant (Fig. S10e-h). *R. irregularis* grew profusely on the root surface and produced complex hyphopodia with many septa (Fig. S10e). Occasionally, roots were invaded by thick penetration pegs (Fig. S10f), followed by massive intercellular colonization in the cortex (Fig. S10g, arrows). Arbuscules were completely missing, however, intercellular hyphae produced many irregular lateral projections (Fig. S10h), presumably representing attempts to invade and colonize cortex cells. Taken together, this pattern of fungal colonization resembles the colonization phenotype of the petunia vpy mutant (Sekhara Reddy et al., 2007).
We next treated colonized roots of all *Medicago* genotypes with phloroglucinol to reveal lignin deposition. As in petunia, both wild type accessions (A17 and R108) did not accumulate any lignin at the point of fungal infection ([Fig. S11a,b](#)). Surprisingly, however, none of the mutants accumulated significant amounts of lignin at infection points ([Fig. S11c-g](#)), including the *vpy*-2 mutant, while prominent lignin deposition was observed along the vasculature in the stele ([Fig. S11h](#)), as in all other genotypes (data not shown). These results indicate that lignin accumulation at AMF infection points is not part of the phenotype of symbiosis mutants in *Medicago*.

**Methods S1 - Supplementary Material and Methods**

*Medicago truncatula* growth conditions and treatments

Seeds of *Medicago truncatula* were scarified with concentrated sulfuric acid for ten minutes, then rinsed five times with sterile water. Subsequently, seeds were surface-sterilised in concentrated Clorox for two minutes, followed by addition of an equal volume of water and an additional minute of sterilization. Then seeds were rinsed with sterile water five times and imbibed at room temp on an orbital shaker for four hours. Then seeds were placed on 0.8% plant agar in a growth chamber (20 ºC, 18 h light) for germination. Seedlings were transferred to sterilized double plastic jars with perlite in the top and B&D nutrient solution (Broughton & Dilworth, 1971) containing 1mM KN03 in the bottom jar, connected by a cotton wick. After one month, seedlings were transferred to nurse plant pots with mycorrhizal jive plants that had been inoculated at least one month before with *R. irregularis*.

**β-1,3-Glucanase immunostaining**

Wild type an *vpy*-3 plants were inoculated with nurse plant inoculum of *R. irregularis*. Root material was fixed for 2h with 2% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde at room temperature. Fixed material was dehydrated and embedded in Lowicryl K4M (www.sigmaaldrich.com) as described (Altman *et al.*, 1984) with the following modifications: embedding involved a series of lowicryl diluted with 95% (v/v) ethanol as follows: 1:2 (vol:vol) for 2h, 1:1 for 2h, 2:1 overnight, followed by twice 100% lowicryl for 2h and polymerization at 55°C. Ultrathin sections (70 nm) were blocked with 20 mM NH4Cl, 20 mM lysine and 20 mM glycine. Rabbit antiserum raised against extracellular glucanase (Beffa *et al.*, 1993) was used at a dilution of 1:5 in blocking agent. Goat-anti-rabbit antibodies coupled to 10 nm gold beads (http://www.bbisolutions.com) served as secondary antibodies. Contrasting was performed with 2% (w/v) uranyl acetate (UO2(CH3COO)2) and lead citrate solution prepared according to (Reynolds, 1963). Controls without the primary antibody did not show any labeling. For quantification of immunogold signal, from each treatment six representative pictures of hypodermal cells from two independent experiments were printed out at the same magnification. A 2D grid of small crosses at regular distances (1 cm) printed on a transparent foil was overlaid and the gold particles were counted in the respective cellular compartments relative to the number of crosses within the same compartment.

**RNA extraction and quantitative real-time RT-PCR**

For gene expression analysis, plants were either inoculated for four weeks with nurse plants ([Tables 1,2](#)), or wild type plants were treated with chitin oligosaccharides or with a *Penicillium* preparation (Thuerig *et al.*, 2005) for 1 and 4h at concentrations of 1 µg/ml and 10 µg/ml, respectively ([Table S9](#)). Frozen petunia roots were placed in 2 mL Eppendorf tubes containing a glass bead and were ground using a ball mill. Total RNA was extracted from the powdered roots according to the protocol of the Direct-zol RNA miniprep kit from Zymo Research.
(https://www.zymoresearch.com), using trizol solution for lysis (38% v/v), saturated phenol (pH 8), 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1M Na-acetate pH 5, and 5% (v/v) glycerol. The Direct-zol RNA kit involves a DNase step to remove genomic DNA during RNA extraction. RNA was used for reverse transcription according to the protocol of the SensiFAST™ cDNA synthesis kit from Bioline. PCR reactions were carried out with 5 µL of 100x diluted cDNA solution, 1 µL of 10 µM forward and reverse primer, 7.5 µL of SensiMix SYBR Hi-ROX (BIO-RAD) and DNase/RNase free water up to 15 µL. The reaction cycle was 95°C for 10 min followed by 45 amplification cycles (95°C for 20s, 64°C for 20s, 72°C for 20s). All samples were analyzed in technical duplicates from six independent replicate plants. Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes. Relative expression values were calculated using the delta-Ct method (Pfaffl, 2001). Data are expressed as -fold changes in relative gene expression (AM/control; vpy/wt) in Tables 1, 2, S3, S8, and S9. Expression values in Tables S4, S5, and S10-S12 represent expression values normalized to actin and GAPDH. Gene expression data were statistically analyzed using two-way ANOVA for interaction between genotype and treatment, followed by one way ANOVA as described below.

**Statistical analysis**

qPCR results were tested by two-way ANOVA with host genotype and mycorrhizal status as the two factors of interest. The p-values of the two factors and their interactions are listed in Table S13. All other data, and the derived gene induction ratios from qPCRs, were treated by one-way ANOVA to test for significance of differences among groups (mutants vs. wild type, AM vs. controls) and the corresponding p-values are also listed in Table S13. Statistical analysis of callose accumulation (Table S1) was performed by Fisher’s exact test for count data to test individual mutants vs. the wild type. This test revealed that callose deposition patterns in the three mutants were not significantly different from the wild type (see below):

```r
> fisher.test(M[,c(1,2)])
Fisher's Exact Test for Count Data
data:  M[, c(1, 2)]
p-value = 0.3824
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
  0.264316   29.001559
sample estimates:
  odds ratio
  2.367128

> fisher.test(M[,c(1,3)])
Fisher's Exact Test for Count Data
data:  M[, c(1, 3)]
p-value = 0.2099
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
  0.06014388   1.60903773
sample estimates:
  odds ratio
  0.3666198

> fisher.test(M[,c(1,4)])
```
Fisher's Exact Test for Count Data
data: M[, c(1, 4)]
p-value = 0.7295
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
0.1069708  3.8126996
sample estimates:
  odds ratio
  0.7129047

**Supplementary Figures and Tables**

**Figure S1. Quantitative phenotypic analysis of vpy mutants.**
Mycorrhizal roots of wild type and vpy mutants were stained with Trypan Blue for quantitative assessment of fungal structures as indicated. Mutants formed infection pegs instead of intracellular coils in the hypodermis, and aborted or malformed structures instead of arbuscules. Shown are the mean + Stdv (n=4). Different letters indicate significant differences (one-way ANOVA).
Figure S2. Confocal analysis of papilla formation in vpy mutants. Mycorrhizal roots of wild type (a), vpy-1 (b), vpy-2 (c), and vpy-3 (d), were stained with basic fuchsin and wheat germ agglutinine (WGA)-Alexa488, and evaluated for cell wall appositions by confocal microscopy. Shown are hyphae (green) and cell walls with papillae (red). Papillae are indicated by arrowheads. (e) Quantification of papilla formation. Representative pictures of penetrated cells as in (a)-(d) were assessed for the number of papillae. Shown are the mean + Stdv of three samples containing at least eight pictures each. Different letters indicate significant differences (one-way ANOVA, n=3). Size bar 25 µm.
Figure S3. Infection phenotype in vpy-3 root cortex cells.
Transmission electron micrographs of mycorrhizal root cortex cells in wild type (a,b,e), and vpy-3 (c,d). (a) An intercellular hypha (IH) has produced an intracellular penetration hypha (PH) that has penetrated the host cell wall (white arrowheads) and that is surrounded by a thin layer of host cell wall material (arrowheads). Several thin-walled fine fungal arbuscule branches are embedded in the cytoplasm of the host (asterisks). The space between the fungal cell wall and the periarbuscular membrane of the host is very thin.
(b) Fully colonised cortex cell containing an arbuscule. Note large trunk hyphae (TH) and thin arbuscular branches (asterisks).
(c) Attempted entry into a vpy-3 cortex cell results in formation of a papilla (arrowheads).
(d) Branched fungal structure in the cortex of a vpy-3 root. In the vicinity of the fungal hyphae (green), multiple small papillae (red) were formed (arrowheads).
(e) Two arbuscules in the cortex of a wild type plant. Fungal hyphae (green) did not elicit any papillae.
(a)-(c) electron micrographs; (d,e) Confocal micrographs. PH, penetration hypha; TH, trunk hypha; x, xylem; IH, intercellular hypha. Scale bars in (a-c), 2 µm; in (d,e) 25 µm.
Figure S4. Cytochemical detection of H$_2$O$_2$ in the cortex of vpy mutants
Mycorrhizal roots of wild type and all vpy alleles were stained with 3,3’-diaminobenzidine (DAB). Shown are representative DAB-stained vpy-2 mutant roots (a-c), and corresponding wild type roots (d-f). All three vpy alleles gave similar results.
(a) Weak background staining in vpy-2. The other vpy mutants and the wild type showed a similar background signal.
(b) Weak signal associated with residual intracellular colonisation in vpy-2 cortex cells (a).
(c) Strong signal in vesicles (v).
(d) In wild type roots, strong staining was observed in arbuscules (a).
(e) Staining was further increased in cells with clumping arbuscules (a’), relative to mature arbuscules (a).
(f) Strong signal in vesicles (v) in the wild type cortex. Size bar 50 µm.
Figure S5. Cytochemical detection of $O_2^-$ in vpy mutants
Wild type and vpy mutants stained with nitroblue tetrazolium (NBT) to detect $O_2^-$. Strongest staining was observed in root tips (a-d) and lateral roots (e) of all genotypes (as indicated), independent of AM inoculation. In colonised areas of all genotypes (as indicated), strongest signal was detected in all fungal structures (f-j). White arrowheads indicate intercellular longitudinal hyphae; yellow arrowheads mark their tips, black arrowheads point to cell wall thickenings in hypodermal cells. m, root meristem; p, penetration peg. Size bars 100 µm in (a-e), 25 µm in (f-j).
Figure S6. Callose accumulation associated with aborted fungal infection of *vpy*-3.
Infection points of mycorrhizal wild type (a,b), and *vpy*-3 (c-h), were evaluated after staining with aniline blue. (a,c,e,g) bright field images, (b,d,f,h) epifluorescence images of the same cells.
(a,b) Successful penetration of a wild type hypodermal cell. No callose can be observed around the hyphal coil.
(c,d) Penetrated cell of a *vpy*-3 mutant root with a deformed hyphal peg but no callose deposition.
(e,f) Aborted fungal entry point into a hypodermal cell with weak callose accumulation (arrowheads).
(g,h) Aborted fungal entry into a hypodermal cell with the accumulation of callose-rich cell wall material (arrowhead). c, hyphal coil; p, penetration peg. Scale bars, 20 µm.
Figure S7. Lack of lignin accumulation in mycorrhizal ram1 mutants

Non-colonised roots (a,b) or roots of colonised wild type (c-f), or colonised ram1 mutants (g,h) were stained with phloroglucinol-HCl (a, c, e, g), or Trypan Blue (b, d, f, h), respectively. (a, b) non-colonised cells, (c,d) intracellular hypodermal coils, (e,f) arbuscules in the wild type, (g,h) infection unit with hyphal coils, and aberrant hyphal colonisation in the cortex (arrows). vasc, vascular bundles in the stele; HPC, hypodermal passage cell; c, hyphal coil; a, arbuscules. size bars 25 µm.
Figure S8. Concerted induction of lignin biosynthetic genes in mycorrhizal vpy mutants. The main biosynthetic route toward the monolignols p-coumaryl, coniferyl, and sinapyl alcohol is shown. Red numbers indicate the induction ratios of the respective gene functions calculated by averaging the induction ratios for the individual lignin-biosynthetic genes in a functional group (isozymes in Table 1), and by averaging the three vpy mutants. Pathway scheme after Vanholme et al. (2010). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; C3H, p-coumarate 3-hydroxylase; HCT, p-hydroxycinnamoyl-CoA:quinate/shikimate p-hydroxycinnamoyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid O-methyltransferase; CAD, cinnamyl alcohol dehydrogenase.

Redrawn with permission from: Vanholme R, Demedts B, Morreel K, Ralph J, Boerjan W. 2010. Lignin biosynthesis and structure. Plant Physiology 153(3): 895-905.
Figure S9. Mycorrhizal colonisation pattern in *M. truncatula* sym mutants

Mycorrhizal roots were stained with Trypan Blue. (a-c) wild type accessions A17 and R108 (as indicated). (d,e) *DMI3* mutant (defective in *CCaMK*), (f,g) *DMI2* mutant (defective in *SYM1*). (a,b) Hyphal coils in hypodermal cells, (c) arbuscules in the cortex, (d,f) fungal hyphae growing on the root surface and attempted entry points with hyphopodia (asterisks), (e,g) cortical colonisation pattern with intercellular hyphae (arrows) (e), and intracellular arbuscules (g). a, arbuscules; c, hyphal coils. Size bars 25 µm.
Figure S10. Mycorrhizal colonisation pattern in *M. truncatula* sym mutants

Mycorrhizal roots were stained with Trypan Blue. (a,b) *ram1* mutants; (c,d) *nsp2-2* mutants; (e-h), *vpy-2* mutants. (a,c) Entry point with hyphal coils; (b) Aberrant arbuscules (arrows) in the *ram1* mutant; (d) widely spaced normal arbuscules with intervening partially developed structures (arrows). (e) Hyphae growing on the root surface with hyphal swellings resembling hyphopodia (asterisks); (f) Fungal entry point with a thick penetration peg; (g,h) profusely growing intercellular hyphae (arrows), occasionally with lateral projections that may represent attempted cell invasion events (inset). a, arbuscules; c, hyphal coils; p, penetration peg. Size bars 25 µm.
Figure S11. Lack of lignin accumulation at fungal entry points in *M. truncatula sym* mutants

Mycorrhizal roots were stained with phloroglucinol. (a,b) Wild type accessions A17 and R108, respectively. (c) *dmi3-1* mutant with attempted infection points represented by hyphal swellings (asterisks), and accumulation of brownish material in host cells (arrows); (d) *DMI2-1* with attempted infection points represented by hyphal swellings (asterisks), and accumulation of brownish material next to the fungal hyphae (arrow); (e) Hyphal coil in a hypodermal cell of a *ram1-1* mutant; (f) Hyphal coil in a hypodermal cell of an *nsp2-2* mutant; (g) Entry point in a *VPI-2* mutant by a hyphal penetration peg (p), and subsequent intercellular hypha; (h) Non-colonised root showing staining of the vasculature in the stele. c, hyphal coils; p, penetration peg; vasc, vasculature. Size bars 25 µm.
Figure S12. Salicylic acid levels in mycorrhizal wild type and vpy-3 roots.
(a) Accumulation of free salicylic acid in wild type *P. hybrida* (white columns), and vpy-3 plants (black columns) after 10 days (10d) and 35 days (35d) with or without inoculation with *R. irregularis* (AM).
(b) Free SA (black columns) and conjugated SA (white columns) in wild type and vpy-3 roots with or without inoculation with *R. irregularis* (AM).
Columns represent means ± Stdv (n=5). There was no significant difference between any of the treatments (one-way ANOVA).
Figure S13. Jasmonic acid levels in mycorrhizal wild type and vpy-3 roots.
(a) Accumulation of free jasmonic acid in wild type *P. hybrida* (white columns), and vpy-3 mutants (black columns) after 10 days (10d) and 35 days (35d) with or without inoculation with *R. irregularis* (AM).
(b) Accumulation of isoleucine-conjugated jasmonic acid (JA-Ile) in wild type *P. hybrida* (white columns), and vpy-3 mutants (black columns) after 10 days (10d) and 35 days (35d) with or without inoculation with *R. irregularis* (AM). Columns represent means + Stdv (n=5). Different letters indicate significant differences (one-way ANOVA).
Figure S14. Immunocytochemical analysis of β-1,3-glucanase in mycorrhizal roots.
Hypodermal cells (a-d), and cortical cells (e-h) of wild type (a,b,e,f), and vpy-3 mutant roots (c,d,g,h). Non-inoculated control plants (a,c,e,g) and mycorrhizal plants (b,d,f,h) were analysed by immunogold staining with antibodies raised against tobacco β-1,3-glucanase. Low levels of immunogold were detected in hypodermal cells (a-d), while label was almost undetectable in cortex cells (e-h). va, plant vacuole; hl, hypodermal layer; 1°, primary cell wall; 2°, secondary cell wall. Scale bars, 250 nm.
Figure S15. Accumulation of β-1,3-glucanase in a cell wall apposition of vpy-3.
Same sample as in Fig. 8d. An aborted fungal penetration hypha (degenerating fungus) grown next to a hypodermal cell (upper right) and a cortex cell (lower left). The hypodermal cell has formed a thick cell wall apposition that exhibits strong immunogold signal. hl, hypodermal layer; 1°, primary cell wall; 2°, secondary cell wall. Scale bar, 500 nm.
Figure S16. Low-magnification overview picture of the sample shown in Fig. S15.
Figure S17. Quantification of β-1,3-glucanase immunogold signal
Immuno-gold signal was quantified in six representative TEM images as shown in Fig. S14 and Fig. S15 from two independent experiments. Gold particles were counted in the cell walls of hypodermal cells and expressed as relative density per unit of section area. Shown are the mean ± Stdv. Different letters indicate significant differences (one-way ANOVA, n=6).

Table S1. Quantification of callose accumulation in vpy mutants
For each genotype at least 30 infection sites with at least one infected cell were chosen randomly. Presence of callose at infection sites was evaluated as shown in Fig. S6 with strong or weak signal at penetration sites into hypodermal cells. The majority of infection sites did not exhibit any callose signal (negative). The column “callose all” refers to the accumulative cases in which either strong or weak signal was detected. The frequency of callose formation was not different in mutants compared to the wild type (Fisher’s Exact Test for count data).
| Gene ID | Forward Primer | Reverse Primer |
|---------|----------------|---------------|
| PAL1    | Peaxi162Scf00123g00096.1 | CAATGGGTGCTAATGGTGAACTTCAT | CCTCATTCTATCTGGAAAAGCTCCCA |
| PAL2    | Peaxi162Scf00488g00074.1 | AAGATTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| PAL3    | Peaxi162Scf00858g00215.1 | CTAGTTGCCAAAGAATTGTGACAT | AGCCTCATTCTATCTGGAAAAGCTCCCA |
| 4CL1    | Peaxi162Scf00314g00086.1 | TCTTCAATTCATCTTCGAAAGCTCCA | CAGAGTTCTACGTCTGCAGCCTTTC |
| 4CL2    | Peaxi162Scf00207g00334.1 | GATCCTGATAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| 4CL3    | Peaxi162Scf00745g00865.1 | CCAAGATTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| 4CL4    | Peaxi162Scf00610g00346.1 | CCAAGATTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| CA4H    | Peaxi162Scf00390g00225.1 | AGAAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| C3H1    | Peaxi162Scf00220g00211.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| C3H2    | Peaxi162Scf00975g00122.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| COMT    | Peaxi162Scf00912g00111.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| CCoAOMT1| Peaxi162Scf00450g00032.1 | CCGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| CCoAOMT2| Peaxi162Scf00166g02023.1 | CCGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| CCoAOMT3| Peaxi162Scf00316g00555.1 | CCGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| HCT     | Peaxi162Scf00220g00211.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| CAD1    | Peaxi162Scf00220g00211.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| CAD2    | Peaxi162Scf00316g00055.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| CCR1    | Peaxi162Scf00332g00433.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| CCR2    | Peaxi162Scf00207g00444.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| FSH1    | Peaxi162Scf00083g00131.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| FSH2    | Peaxi162Scf00083g00131.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| Actin7  | Peaxi162Scf00207g00444.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |
| GAPDH   | Peaxi162Scf00763g00325.1 | GAGTTGCAGCTTTTGAAGACGAGTT | ACCTCATTCTATCTGGAAAAGCTCCCA |

Table S2. Primers used for qRT-PCR of lignin-related genes

Sequence identifiers and qRT-PCR primers for genes encoding predicted lignin biosynthetic enzymes. Gene IDs refer to gene names from the Solanaceae Genome Network database (https://solgenomics.net/) using the JBrowse function in the Petunia axillaris genome (https://solgenomics.net/organism/Petunia_axillaris/genome).
Table S3. Induction of lignin-related genes in vpy mutants vs. wild type either in the mycorrhizal or non-mycorrhizal context.

Induction of lignin biosynthetic genes was determined by qRT-PCR with actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference genes. Values represent fold induction ratios derived by dividing the expression values of mutants by the values from the wild type, either in mycorrhizal (left) or control conditions (right). All expression values represent the average of six biological replicates. Color shading represents induction >2-fold (yellow), >4-fold (orange), and >8-fold (red). Data represent two independent experiments, one with only vpy-1 vs. wild type (asterisks), and one with vpy-2 and vpy-3 vs. wild type. Significant induction ratios are indicated by bold type font (one-way ANOVA, n=6).

|          | AM(mut) / AM(wt) | c(mut) / c(wt) |
|----------|------------------|----------------|
|          | vpy1* | vpy2 | vpy3 | vpy1* | vpy2 | vpy3 |
| PAL-1    | 1.95  | 4.86 | 5.73 | 1.27  | 1.29 | 0.94 |
| PAL-2    | 2.01  | 3.88 | 5.85 | 1.28  | 1.67 | 1.52 |
| PAL-3    | 3.62  | 6.25 | 4.01 | 2.68  | 1.68 | 1.28 |
| 4CL-1    | 5.49  | 4.11 | 6.16 | 0.82  | 1.60 | 1.14 |
| 4CL-2    | 5.21  | 3.37 | 5.39 | 0.81  | 1.52 | 1.19 |
| 4CL-3    | 15.85 | 18.59| 30.85| 0.35  | 1.05 | 0.79 |
| 4CL-4    | 6.76  | 21.71| 16.07| 1.07  | 0.49 | 0.98 |
| CA4H     | 4.36  | 5.67 | 3.87 | 0.90  | 0.98 | 1.01 |
| C3H-1    | 5.00  | 5.63 | 6.24 | 0.51  | 0.77 | 0.62 |
| C3H-2    | 3.41  | 3.16 | 5.10 | 0.78  | 1.90 | 1.60 |
| COMT     | 3.26  | 7.00 | 8.19 | 0.61  | 1.58 | 1.39 |
| CCoAOMT-1| 6.82  | 4.39 | 4.24 | 0.59  | 1.88 | 1.52 |
| CCoAOMT-2| 3.06  | 4.89 | 7.20 | 1.15  | 1.80 | 1.53 |
| CCoAOMT-3| 3.21  | 17.65| 37.97| 3.73  | 0.96 | 1.45 |
| HCT      | 2.42  | 5.84 | 12.15| 2.70  | 0.91 | 1.45 |
| CAD1     | 5.59  | 6.58 | 6.22 | 1.47  | 0.96 | 1.08 |
| CAD2     | 8.20  | 15.16| 26.65| 0.74  | 0.86 | 0.89 |
| CCR1     | 6.18  | 21.26| 30.11| 0.83  | 0.91 | 1.03 |
| CCR2     | 6.18  | 6.64 | 5.80 | 1.06  | 0.88 | 1.17 |
| F5H2     | 4.31  | 4.78 | 14.36| 1.01  | 1.33 | 1.18 |

Yellow: induction>2; orange: induction>4; red: induction>8; bold: p<0.05
Table S4. Expression values for lignin-related genes for the experiment on \textit{vpy-1} from the genes for which induction ratios were calculated in Table 1 and Table S3.

| Gene      | non-AM \(\text{wt} \) | non-AM \(\text{vpy-1} \) | AM \(\text{wt} \) | AM \(\text{vpy-1} \) |
|-----------|------------------------|--------------------------|-----------------|-------------------|
| PAL-1     | 0.60                   | 0.76                     | 0.62            | 1.53              |
| PAL-2     | 1.51                   | 1.93                     | 3.85            | 9.87              |
| PAL-3     | 0.19                   | 0.51                     | 0.24            | 2.33              |
| 4CL-1     | 0.31                   | 0.25                     | 0.88            | 3.99              |
| 4CL-2     | 0.10                   | 0.08                     | 0.19            | 0.78              |
| 4CL-3     | 0.02                   | 0.01                     | 0.02            | 0.11              |
| 4CL-4     | 0.04                   | 0.04                     | 0.03            | 0.22              |
| CA4H      | 0.55                   | 0.50                     | 0.53            | 2.09              |
| C3H-1     | 0.11                   | 0.05                     | 0.17            | 0.42              |
| C3H-2     | 0.00                   | 0.00                     | 0.00            | 0.01              |
| COMT      | 0.19                   | 0.12                     | 0.21            | 0.42              |
| CCoAOMT-1 | 0.34                   | 0.20                     | 0.35            | 1.41              |
| CCoAOMT-2 | 0.51                   | 0.59                     | 0.60            | 2.12              |
| CCoAOMT-3 | 0.00                   | 0.00                     | 0.00            | 0.01              |
| HCT       | 0.00                   | 0.00                     | 0.00            | 0.01              |
| CAD1      | 0.19                   | 0.27                     | 0.23            | 1.86              |
| CAD2      | 0.06                   | 0.04                     | 0.06            | 0.38              |
| CCR1      | 0.07                   | 0.06                     | 0.08            | 0.41              |
| CCR2      | 0.16                   | 0.17                     | 0.13            | 0.84              |
| F5H2      | 0.09                   | 0.09                     | 0.09            | 0.39              |
Table S5. Expression values for lignin-related genes for the experiment on *vpy*-2 and *vpy*-3 from the genes for which induction ratios were calculated in Table 1 and Table S3.

|                | non-AM |       |       | AM    |       |       |
|----------------|--------|-------|-------|-------|-------|-------|
|                | **wt** | **vpy-2** | **vpy-3** | **wt** | **vpy-2** | **vpy-3** |
| PAL-1          | 0.37   | 0.48  | 0.35  | 0.44  | 2.74  | 2.37  |
| PAL-2          | 1.52   | 2.53  | 2.30  | 3.14  | 20.24 | 27.86 |
| PAL-3          | 0.20   | 0.34  | 0.26  | 0.27  | 2.83  | 1.38  |
| 4CL-1          | 0.37   | 0.59  | 0.42  | 0.69  | 4.49  | 4.81  |
| 4CL-2          | 0.10   | 0.14  | 0.11  | 0.13  | 0.68  | 0.86  |
| 4CL-3          | 0.02   | 0.02  | 0.01  | 0.02  | 0.33  | 0.41  |
| 4CL-4          | 0.03   | 0.01  | 0.03  | 0.02  | 0.21  | 0.32  |
| CA4H           | 0.96   | 0.94  | 0.97  | 1.01  | 5.63  | 3.95  |
| C3H-1          | 0.30   | 0.23  | 0.19  | 0.36  | 1.56  | 1.40  |
| C3H-2          | 0.01   | 0.02  | 0.01  | 0.01  | 0.06  | 0.08  |
| COMT           | 0.24   | 0.37  | 0.33  | 0.45  | 5.00  | 5.16  |
| CCoAOMT-1      | 0.20   | 0.38  | 0.31  | 0.31  | 2.54  | 1.99  |
| CCoAOMT-2      | 1.03   | 1.85  | 1.57  | 1.23  | 10.86 | 13.58 |
| CCoAOMT-3      | 0.00   | 0.00  | 0.00  | 0.00  | 0.02  | 0.05  |
| HCT            | 0.00   | 0.00  | 0.00  | 0.00  | 0.02  | 0.07  |
| CAD1           | 0.09   | 0.09  | 0.10  | 0.21  | 1.33  | 1.42  |
| CAD2           | 0.17   | 0.15  | 0.15  | 0.19  | 2.51  | 4.56  |
| CCR1           | 0.03   | 0.03  | 0.03  | 0.04  | 0.86  | 1.37  |
| CCR2           | 0.10   | 0.09  | 0.11  | 0.12  | 0.70  | 0.81  |
| F5H2           | 0.03   | 0.04  | 0.04  | 0.03  | 0.20  | 0.53  |
Table S6. *M. truncatula* mutants used in this study

| Mutant          | Affected gene | Genetic background | Reference                                      |
|-----------------|---------------|--------------------|------------------------------------------------|
| dmi2-1          | SYMRK         | A17                | Endre et al., 2002                             |
| dmi3-1          | CCAMK         | A17                | Lévy et al., 2004; Mitra et al., 2004          |
| ram1-1          | RAM1          | A17                | Gobbato et al., 2012                           |
| nsp2-2          | NSP           | A17                | Kalo et al., 2005                              |
| vpy-2 (NF6898.86) | VAPYRIN      | R108               | Liu et al., 2019                               |

Endre G, Kereszt A, Kevei Z, Mihacea S, Kaló P, Kiss GB. 2002. A receptor kinase gene regulating symbiotic nodule development. *Nature* 417(6892): 962-966.

Gobbato E, Marsh JF, Vernie T, Wang E, Maillet F, Kim J, Miller JB, Sun J, Bano SA, Ratet P, et al. 2012. A GRAS-type transcription factor with a specific function in mycorrhizal signaling. *Current Biology* 22(23): 2236-2241.

Kalo P, Gleason C, Edwards A, Marsh J, Mitra RM, Hirsch S, Jakab J, Sims S, Long SR, Rogers J, et al. 2005. Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. *Science* 308(5729): 1786-1789.

Lévy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ané JM, Lauber E, Bisseling T, et al. 2004. A putative Ca2+ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* 303(5662): 1361-1364.

Liu CW, Breakspear A, Stacey N, Findlay K, Nakashima J, Ramakrishnan K, Liu MX, Xie F, Endre G, de Carvalho-Niebel F, et al. 2019. A protein complex required for polar growth of rhizobial infection threads. *Nature Communications* 10: 2848.

Mitra RM, Gleason CA, Edwards A, Hadfield J, Downie JA, Oldroyd GED, Long SR. 2004. A Ca2+/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. *Proceedings of The National Academy of Sciences of The United States of America* 101(13): 4701-4705.
Table S7. Primers used for qRT-PCR of PR genes

Sequence identifiers and qRT-PCR primers for genes encoding predicted PR proteins. Gene IDs refer to gene names from the Solanaceae Genome Network database ([https://solgenomics.net/](https://solgenomics.net/)) using the JBrowse function in the *Petunia axillaris* genome ([https://solgenomics.net/organism/Petunia_axillaris/genome](https://solgenomics.net/organism/Petunia_axillaris/genome)).
Table S8. Induction of pathogenesis-related (PR) genes in mutants vs. wild type either in the mycorrhizal or non-mycorrhizal context.
Induction of PR genes was determined by qRT-PCR with actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference genes. Values represent -fold induction ratios derived by dividing the expression values of mutants by the values from the wild type, either in mycorrhizal (left) or control conditions (right). All expression values represent the average of six biological replicates. Color shading represents induction >2-fold (yellow), >4-fold (orange), and >8-fold (red). Data represent two independent experiments, one with only $vpy\text{-}1$ vs. wild type (asterisks), and one with $vpy\text{-}2$ and $vpy\text{-}3$ vs. wild type. Significant induction ratios are indicated by bold type font (one-way ANOVA, n=6).

|       | 1h       |        | 4h       |        |
|-------|----------|--------|----------|--------|
|       | Chit     | Pen    | Chit     | Pen    |
| PR2a  | 1.38     | 2.93   | 0.61     | 0.82   |
| PR2b  | 1.52     | 0.88   | 0.82     | 0.50   |
| PR2c  | 1.38     | 1.35   | **2.61** | **3.37** |
| PR3   | 1.91     | 1.33   | 0.93     | **1.78** |
| PR4b  | 0.89     | 1.64   | 1.15     | **2.44** |
| PR4c  | **2.04** | 0.88   | 0.97     | 0.92   |
| PR4d  | 1.61     | 1.61   | **5.45** | **5.02** |
| PR5a  | 1.45     | 2.21   | 1.54     | **3.26** |
| PR6a  | 0.73     | 0.61   | 0.92     | **2.84** |
| PR7   | **2.99** | 1.02   | 0.94     | 1.34   |
| PR9   | 2.16     | 1.22   | 1.27     | 1.17   |
| PR14  | 1.33     | 0.87   | 1.02     | 1.07   |
| PR17  | 1.55     | 0.64   | 0.99     | **1.96** |

Yellow: induction>2; orange: induction>4; bold: p<0.05

Table S9. Induction of pathogenesis-related (PR) genes in wild type plants treated with fungal elicitor preparations.
Induction of PR genes was determined by qRT-PCR with actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference genes. Values represent -fold induction ratios derived by dividing the expression values of roots treated with chitin hydrolysate (Chit) or Penicillium preparation (Pen) for 1h or 4h by the values of control roots. All expression values represent the average of six biological replicates. Color shading represents induction >2-fold (yellow), and >4-fold (orange). Significant induction ratios are indicated by bold type font (one-way ANOVA, n=6).
Table S10. Expression values for the experiment on \textit{vpy-1} from the genes for which induction ratios were calculated in Table 2 and Table S8.

|     | non-AM |          | AM     |
|-----|--------|----------|--------|
|     | wt     | \textit{vpy-1} | wt     | \textit{vpy-1} |
| PR2a | 0.0006 | 0.0005   | 0.0008 | 0.0098 |
| PR2b | 0.0002 | 0.0005   | 0.0003 | 0.0039 |
| PR2c | 0.2096 | 0.1985   | 0.3112 | 0.3707 |
| PR3  | 0.0169 | 0.0090   | 0.0197 | 0.0593 |
| PR4b | 0.7377 | 0.8976   | 1.5068 | 8.6525 |
| PR4c | 0.0012 | 0.0011   | 0.0011 | 0.0094 |
| PR4d | 0.2050 | 2.0049   | 1.1415 | 4.7825 |
| PR5a | 2.0615 | 4.9515   | 13.7566 | 5.6603 |
| PR6a | 0.0222 | 0.1489   | 0.0399 | 0.9097 |
| PR7  | 0.0099 | 0.0028   | 0.0421 | 0.0762 |
| PR9  | 0.0013 | 0.0011   | 0.0092 | 0.0270 |
| PR14 | 0.0024 | 0.0020   | 0.0060 | 0.0298 |
| PR17 | 0.2138 | 0.3355   | 0.2889 | 3.0270 |

Table S11. Expression values for the experiment on \textit{vpy-2} and \textit{vpy-3} from the genes for which induction ratios were calculated in Table 2 and Table S8.

|     | non-AM | \textit{vpy-2} | \textit{vpy-3} | AM     |
|-----|--------|---------------|---------------|--------|
|     | wt     | \textit{vpy-2} | \textit{vpy-3} | wt     | \textit{vpy-2} | \textit{vpy-3} |
| PR2a | 0.0008 | 0.0005       | 0.0018       | 0.0020 | 0.0158 | 0.0235 |
| PR2b | 0.0003 | 0.0001       | 0.0004       | 0.0004 | 0.0025 | 0.0103 |
| PR2c | 0.5561 | 0.6429       | 0.3387       | 0.7008 | 0.7030 | 0.2983 |
| PR3  | 0.0119 | 0.0156       | 0.0196       | 0.0165 | 0.0782 | 0.0609 |
| PR4b | 0.3502 | 0.2093       | 1.0678       | 1.9080 | 8.7328 | 13.8989 |
| PR4c | 0.0032 | 0.0033       | 0.0042       | 0.0035 | 0.0355 | 0.0580 |
| PR4d | 0.1799 | 0.3843       | 0.7605       | 0.9921 | 1.0316 | 1.2303 |
| PR5a | 3.5041 | 3.4324       | 9.5838       | 12.1965 | 3.4931 | 10.8562 |
| PR6a | 0.0011 | 0.0183       | 0.0179       | 0.0152 | 0.6182 | 0.0281 |
| PR7  | 0.0050 | 0.0050       | 0.0074       | 0.0260 | 0.1687 | 0.0522 |
| PR9  | 0.0015 | 0.0020       | 0.0020       | 0.0079 | 0.0128 | 0.0532 |
| PR14 | 0.0038 | 0.0024       | 0.0048       | 0.0114 | 0.0527 | 0.0949 |
| PR17 | 0.1266 | 0.2012       | 0.2844       | 0.1771 | 1.7394 | 0.7017 |
Table S12. Expression values from the experiment with wild type plants treated with elicitors from genes for which induction ratios were calculated in Table S9.

|       | 1h     |        |        | 4h     |        |        |
|-------|--------|--------|--------|--------|--------|--------|
|       | cont   | CHIT   | PEN    | cont   | CHIT   | PEN    |
| PR2a  | 0.004  | 0.006  | 0.013  | 0.016  | 0.010  | 0.013  |
| PR2b  | 0.001  | 0.002  | 0.001  | 0.004  | 0.003  | 0.002  |
| PR2c  | 1.825  | 2.514  | 2.467  | 6.542  | 17.080 | 22.041 |
| PR3   | 0.006  | 0.011  | 0.007  | 0.009  | 0.008  | 0.015  |
| PR4b  | 1.126  | 1.006  | 1.848  | 1.971  | 2.269  | 4.800  |
| PR4c  | 0.002  | 0.004  | 0.002  | 0.007  | 0.006  | 0.006  |
| PR4d  | 0.679  | 1.091  | 1.091  | 1.709  | 9.311  | 8.569  |
| PR5a  | 5.513  | 7.994  | 12.161 | 19.895 | 30.722 | 64.844 |
| PR6a  | 0.579  | 0.423  | 0.356  | 1.023  | 0.937  | 2.906  |
| PR7   | 0.028  | 0.083  | 0.028  | 0.062  | 0.058  | 0.083  |
| PR9   | 0.005  | 0.011  | 0.006  | 0.012  | 0.016  | 0.015  |
| PR14  | 0.041  | 0.055  | 0.036  | 0.050  | 0.051  | 0.053  |
| PR17  | 0.173  | 0.269  | 0.110  | 0.204  | 0.203  | 0.402  |

Table S13. p-values from statistical analyses and interaction report from two-way ANOVA tests

(see separate Excel sheet)