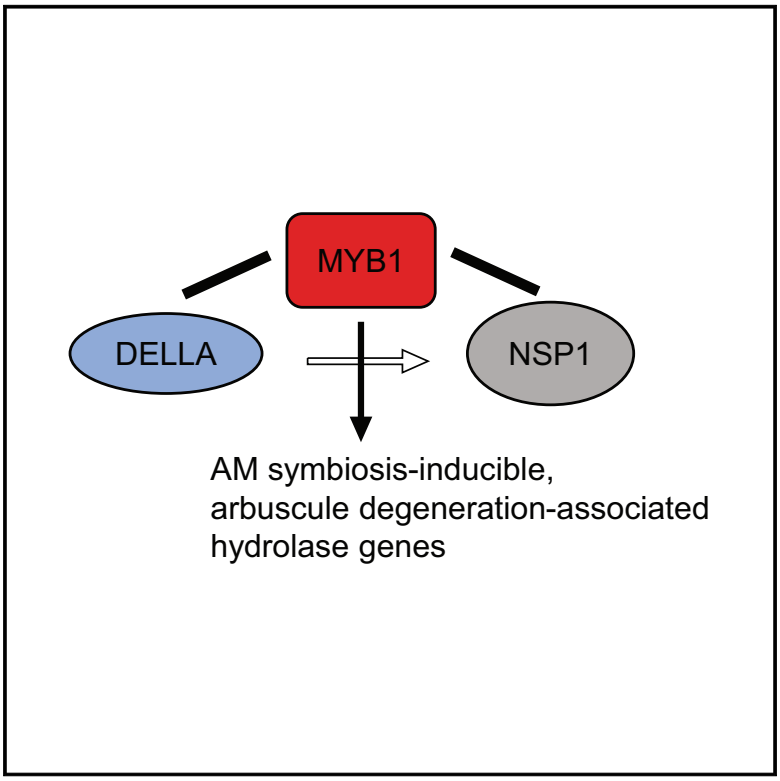
Report

Current Biology

A Transcriptional Program for Arbuscule Degeneration during AM Symbiosis Is Regulated by MYB1

Graphical Abstract



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In Brief

During AM symbiosis, the root cortical cells construct and later disassemble a membrane and matrix around the fungal symbiont. Floss et al. identify a transcriptional program associated with arbuscule degeneration and reveal that expression of degeneration-associated hydrolase genes is regulated by MYB1 in association with NSP1 and DELLAs.

Highlights

- A transcriptional program associated with arbuscule degeneration during AM symbiosis
- Arbuscule degeneration-associated genes encoding hydrolases are regulated by MYB1
- *della* double and triple mutants reveal that DELLAs influence arbuscule degeneration
- MYB1-regulated gene expression requires NSP1 and is enhanced by DELLAs

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Report

A Transcriptional Program for Arbuscule Degeneration during AM Symbiosis Is Regulated by MYB1

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SUMMARY

During the endosymbiosis formed between plants and arbuscular mycorrhizal (AM) fungi, the root cortical cells are colonized by branched hyphae called arbuscules, which function in nutrient exchange with the plant [1]. Despite their positive function, arbuscules are ephemeral structures, and their development is followed by a degeneration phase, in which the arbuscule and surrounding periarbuscular membrane and matrix gradually disappear from the root cell [2, 3]. Currently, the root cell's role in this process and the underlying regulatory mechanisms are unknown. Here, by using a *Medicago truncatula pt4* mutant in which arbuscules degenerate prematurely [4], we identified arbuscule degeneration-associated genes, of which 38% are predicted to encode secreted hydrolases, suggesting a role in disassembly of the arbuscule and interface. Through RNAi and analysis of an insertion mutant, we identified a symbiosis-specific MYB-like transcription factor (MYB1) that suppresses arbuscule degeneration in *mtpt4*. In *myb1*, expression of several degeneration-associated genes is reduced. Conversely, in roots constitutively overexpressing *MYB1*, expression of degeneration-associated genes is increased and subsequent development of symbiosis is impaired. MYB1-regulated gene expression is enhanced by DELLA proteins and is dependent on NSP1 [5], but not NSP2 [6]. Furthermore, MYB1 interacts with DELLA and NSP1. Our data identify a transcriptional program for arbuscule degeneration and reveal that its regulators include MYB1 in association with two transcriptional regulators, NSP1 and DELLA, both of which function in preceding phases of the symbiosis. We propose that the combinatorial use of transcription factors enables the sequential expression of transcriptional programs for arbuscule development and degeneration.

RESULTS AND DISCUSSION

Arbuscules are terminally differentiated hyphae that develop in the root cortical cells and are housed within a matrix-filled apoplastic compartment delimited by the plant-derived periarbuscular membrane (PAM) [2]. The resulting symbiotic interface is central for nutrient exchange in the symbiosis, and several plant genes required for its development [7, 8] and function [4, 9–12] have been identified [13, 14]. A symbiosis-signaling pathway and downstream transcription factors CYCLOPS, RAM1, and RAD1 regulate their expression [15–20]. Additionally, gibberellic acid signaling modulates arbuscule development via DELLA proteins, which positively regulate expression of *RAM1* and *RAD1* [19, 21, 22]. By contrast, a mechanistic understanding of the processes underlying arbuscule degeneration is lacking. The first morphological signs of degeneration occur in the fine branches of the arbuscule with loss of cytoplasm and collapse of the hyphal walls. Septa appear in the thick branches or the trunk and eventually occlude cytoplasmic continuity with the intercellular hyphae [2, 23]. The arbuscule remnants, including the surrounding matrix and PAM, gradually disappear from the cell. Disassembly of this complex structure is likely mediated by the root cell, which remains viable throughout this process. In *M. truncatula* and rice phosphate transporter mutants, arbuscule degeneration occurs prematurely, indicating that phosphate transporter function at the PAM influences arbuscule lifespan [4, 9].

At a molecular level, arbuscule degeneration is difficult to study, because degeneration occurs asynchronously within the arbuscule population; however, the phosphate transporter mutant, *M. truncatula pt4*, offers an enriched source of degenerating arbuscules and a platform from which to study this phase of the symbiosis. To gain insight into the plant transcriptome associated with arbuscule degeneration, we compared transcript

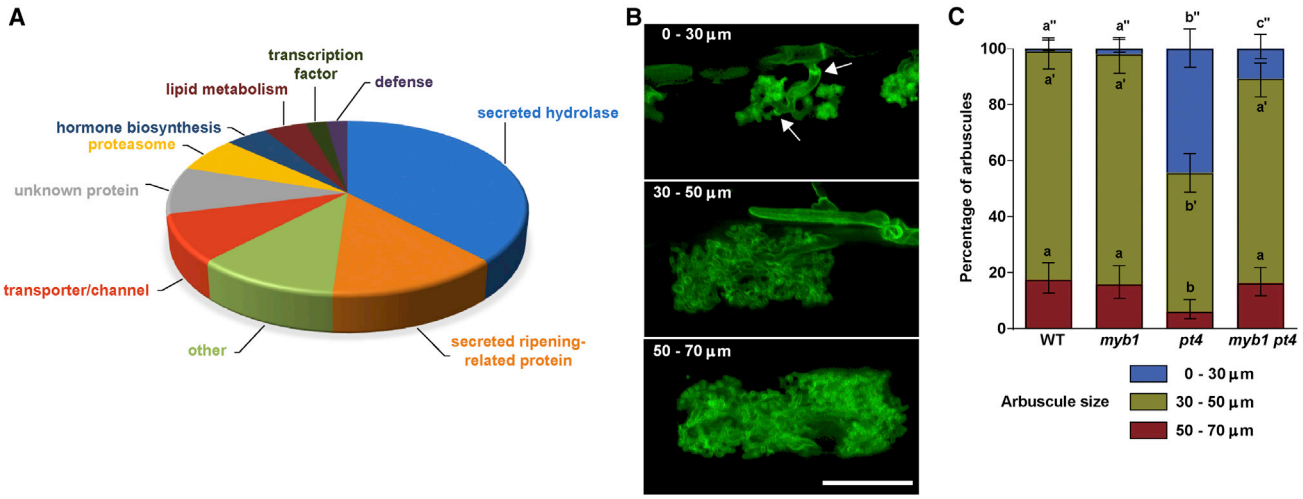

Figure 1. Transcript Profiling of *pt4-1* Reveals a *M. truncatula* Transcriptional Program for Arbuscule Degeneration

(A) GO term classification of genes whose transcript levels are elevated in *pt4-1* mycorrhizal roots relative to A17. Forty-five genes showing a log2 difference (*pt4-1*- A17) > 1, p value < 0.01 are shown.

(B and C) Arbuscule size distribution in arbuscule populations in wild-type (WT), *myb1*, and *pt4-1* (*pt4*) single mutants and the *myb1 pt4* double mutant.

(B) Laser-scanning confocal microscope images of arbuscules from *G. versiforme* in *M. truncatula* roots are shown. Roots were stained with wheat-germ agglutinin (WGA)-Alexa Fluor 488. Arbuscules were classified based on length (0–30 μm: small, degenerating; 30–50 μm: medium; 50–70 μm: large). All small, degenerating arbuscules contain septa (arrows). The scale bar represents 25 μm in each image.

(C) Arbuscule populations at 6 days post-physical contact with primed *G. versiforme* spores are shown. Data represent averages ± SE from between five and ten randomly selected infections from three biological replicate samples of wild-type (189 arbuscules), *myb1* (152 arbuscules), *pt4-5* (198 arbuscules), and *myb1 pt4-5* (204 arbuscules). Different letters indicate significant differences ($p \leq 0.05$). SE bars are not symmetric because of the inverse-logit transformation used to revert model estimates for each group into expected proportions.

Additional data are in Table S1 and Figures S1 and S2.

profiles of colonized *pt4* mutant roots with those of wild-type (A17) at 11 days post-contact with spores. Using *M. truncatula* Affymetrix GeneChips, we identified 91 genes that are differentially expressed in *pt4-1* relative to wild-type (≥2-fold difference; $p \leq 0.01$), with 45 genes showing higher transcript levels in *pt4-1*. Of these 45 genes, 51% are predicted to encode secreted proteins annotated either as hydrolases or ripening-related proteins, including nine cysteine proteases, four chitinases, a triacylglycerol lipase (TGL), a S1/P1 nuclease, and a phosphatase. Data from the *M. truncatula* gene expression atlas indicate that 31 of these 45 genes are induced during arbuscular mycorrhizal (AM) symbiosis and five of them are predicted to be conserved for AM symbiosis [24] (Figure 1A; Table S1). By contrast, among the genes showing lower expression in *pt4-1* relative to A17, only 11% were predicted to encode hydrolases.

Differential expression of a selection of the predicted hydrolase genes was confirmed in a second *pt4* allele, *pt4-2* [4] (Figure S1A). In addition, we demonstrated that *cysteine protease 3* (*CP3*) promoter activity was enhanced in colonized *pt4-2* roots relative to wild-type roots (Figures S1B and S1C). Thus, arbuscule degeneration is accompanied by increased expression of a distinct set of AM-induced genes potentially involved in degradation of the matrix and membranes of the periarbuscular interface as well as the chitinaceous walls of the arbuscule.

In parallel, an RNAi-based screen to identify genes involved in symbiosis [21, 25, 26] provided initial evidence that a construct targeting a MYB family transcription factor, Medtr7g068600 (MYB1) [27, 28], suppressed arbuscule degeneration in *pt4-1* (Figure S2A). Suppression of the *pt4* phenotype was observed consistently in several RNAi experiments, but as detailed quantitative analyses of AM symbiosis are difficult in the composite plant system, we searched for a stable *myb1 Tnt1* mutant to enable further analyses. A loss-of-function allele was not available, but a line with an insertion in the first intron showed a reduction in *MYB1* transcripts (Figures S2B and S2C). We anticipated that *myb1* would show increased colonization, but whereas this trend was apparent in several experiments, it was not consistently statistically significant (Figure S2D). To evaluate the ability of *myb1* to suppress the *pt4* phenotype, we generated a *myb1 pt4-5* double mutant. For these experiments, we used the *pt4-5* allele, as it shares the same genetic background as *myb1* (R108 background) [29]. Quantitative analysis of the arbuscule populations revealed that the proportion of degenerating arbuscules (defined as small arbuscules that contain septa) was reduced significantly in *myb1 pt4-5* as compared to *pt4-5*, whereas the proportion of mid-size and large arbuscules was significantly increased (Figures 1B and 1C). These data are consistent with the initial mutant screen and indicate that *myb1* suppresses arbuscule degeneration in *pt4-5*. As observed for the colonization levels, statistically significant differences in the size distribution of arbuscules in *myb1* relative to wild-type roots were not observed. This is probably because *myb1* is not a loss-of-function allele, and therefore, the effects of reduced function are discernable only in a more permissive background, such as *pt4*, where the base level of degenerating arbuscules is high and small shifts in the population are more easily observed.

MYB1 belongs to a large family of MYB-like transcription factors that includes the *Arabidopsis thaliana* PHR1 and PHL1

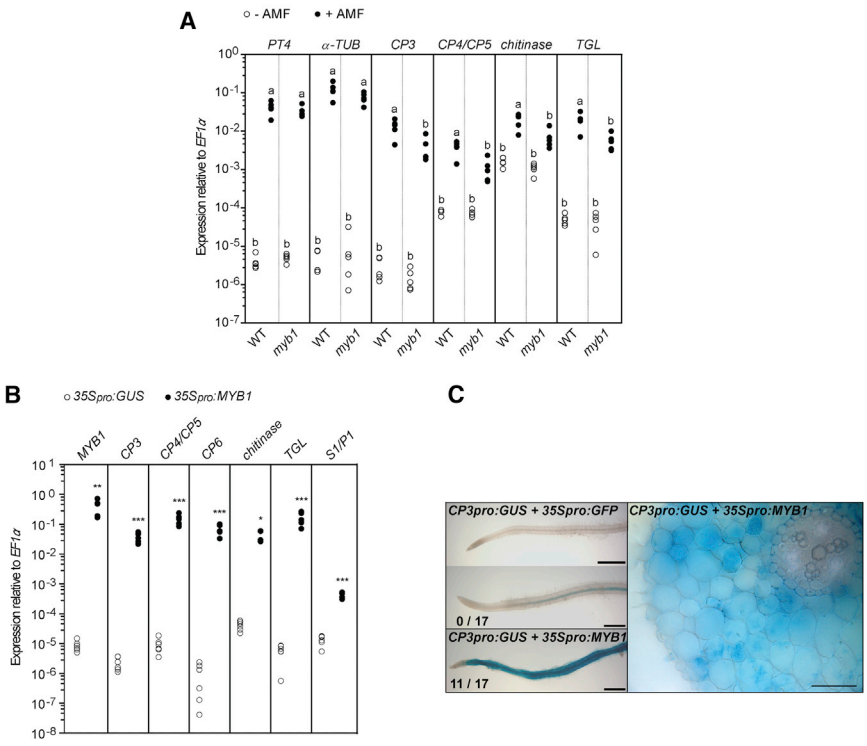

Figure 2. Constitutive Overexpression of *MYB1* Is Sufficient to Induce Expression of Degeneration-Associated Hydrolase Genes

(A) Transcript levels of genes encoding hydrolases in WT and *myb1* roots either mock inoculated (−AMF) or colonized by *G. versiforme* (+AMF) at 26 days post-planting (dpp). Expression values are relative to *EF1α*. *PT4*, and *G. versiforme α-tubulin* (*α-TUB*) transcripts provide an indication of colonization levels. Relative expression values of less than 10^{-5} are considered to be background levels as *G. versiforme* transcripts are not present in mock-inoculated samples. In colonized *myb1* roots, *PT4* and *G. versiforme α-tubulin* expression, respectively, does not differ significantly from that of wild-type roots. Data shown are the individual values of greater than or equal to four biological replicates. Statistical analysis was performed with ANOVA followed by Tukey's honestly significant difference (HSD) test. Different letters indicate significant differences ($p \leq 0.05$).

(B) Transcript levels of genes encoding hydrolases in non-colonized *M. truncatula* (A17) roots expressing either *35Spro:GUS* (vector control) or *35Spro:MYB1* at 20 dpp. Data are individual values from greater than or equal to five biological replicates. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; t test comparing transcript levels in *35Spro:GUS* and *35Spro:MYB1* roots.

(C) GUS activity in non-colonized *M. truncatula* (A17) roots co-expressing *cysteine protease 3* promoter:*GUS* (*CP3pro:GUS*) with either a vector control (*35Spro:GFP*) or with *35Spro:MYB1* at 16 dpp. Roots co-transformed with *CP3pro:GUS* and *35Spro:MYB1* show strong GUS activity throughout the root cortex. Numbers of plants with detectable GUS activity throughout the root cortex per total number of plants examined are indicated. The scale bars represent 1 mm (left panels) and 50 μm (right panel). Additional data are in Figure S3.

genes (Figure S2E), which control phosphate starvation-inducible gene expression [30, 31], and *Lotus japonicus MAMI*, which influences root growth [32]. Previous transcriptome analyses indicate that *MYB1* transcripts are detected only in mycorrhizal roots, specifically in cells containing arbuscules and adjacent cortical cells [28, 33, 34] (*M. truncatula* gene expression atlas; https://mtgea.noble.org/v3/), and we confirmed this spatial expression pattern through analysis of a *MYB1* promoter:*GUS* fusion (Figure S3A). Analysis of *MYB1* transcript levels in roots at 2, 3, and 4 weeks post planting (wpp) indicate that *Myb1* transcripts increase with increasing colonization as do those of *PT4* (Figure S3B). Additionally, we found that *MYB1* transcripts were not detectable in colonized roots of *ram1*, and in contrast to many AM symbiosis-induced genes, *MYB1* transcripts were not detected in non-colonized roots overexpressing a dominant DELLA1 allele (Figures S3C and S3D) [35]. These data suggest that *MYB1* gene expression is regulated by a pathway that differs from many of the symbiosis-induced genes reported to date.

Based on the phenotype of *myb1 pt4* and the prediction that MYB1 is a transcription factor, we hypothesized that MYB1 regulates arbuscule degeneration-associated gene expression. To test this hypothesis, we analyzed expression of a selection of degeneration-associated genes in *myb1* and wild-type roots during symbiosis. Transcript levels of *PT4* and *G. versiforme α-tubulin*, which serve as molecular markers of symbiosis, did not differ significantly in *myb1* and wild-type, indicating that colonization is similar in the two lines. However, transcripts of the symbiosis-induced *CP3*, *CP4/CP5*, *chitinase*, and *TGL* genes were significantly lower in *myb1* relative to wild-type (Figure 2A). Conversely, constitutive overexpression of *MYB1* in *M. truncatula* roots in the absence of the fungus resulted in significant increases in *CP3*, *CP4/CP5*, *CP6*, *chitinase*, *TGL*, and *S1P1* transcript levels (Figure 2B). Furthermore, 65% of roots co-transformed with *CP3* promoter:*GUS* (*CP3pro:GUS*) and CaMV 35S promoter:*MYB1* (*35Spro:MYB1*) constructs showed strong GUS staining throughout the root cortex, which was not observed in roots co-transformed with *CP3pro:GUS* and a *35Spro:GFP* vector control (Figure 2C). Together, these data indicate that *MYB1* expression is sufficient to induce expression of these arbuscule degeneration-associated genes in roots in the absence of an AM fungus. In contrast, genes associated with arbuscule development are not induced in response to overexpression of *MYB1* (Figure S3E). Thus, data from both the *myb1* mutant and from overexpression of *MYB1* indicate that MYB1 regulates expression of arbuscule degeneration-associated hydrolase genes. Subsequent inoculation experiments revealed that overexpression of *MYB1* had a negative impact on fungal proliferation within the cortex (Figures 3A and 3B). In roots constitutively overexpressing *MYB1*, 43% of the infections lacked arbuscules entirely (Figure 3A, middle panel) whereas 14% showed degenerating arbuscules reminiscent of those observed in *pt4* (Figure 3A, lower panel). Overall, colonization levels were 50% lower and fungal infections were 62.5% shorter in *MYB1* overexpressors relative to the control roots (Figure 3B). Transcript levels of *M. truncatula PT4*, *lectin 5*, and *G. versiforme α-tubulin* genes also indicated reduced colonization levels

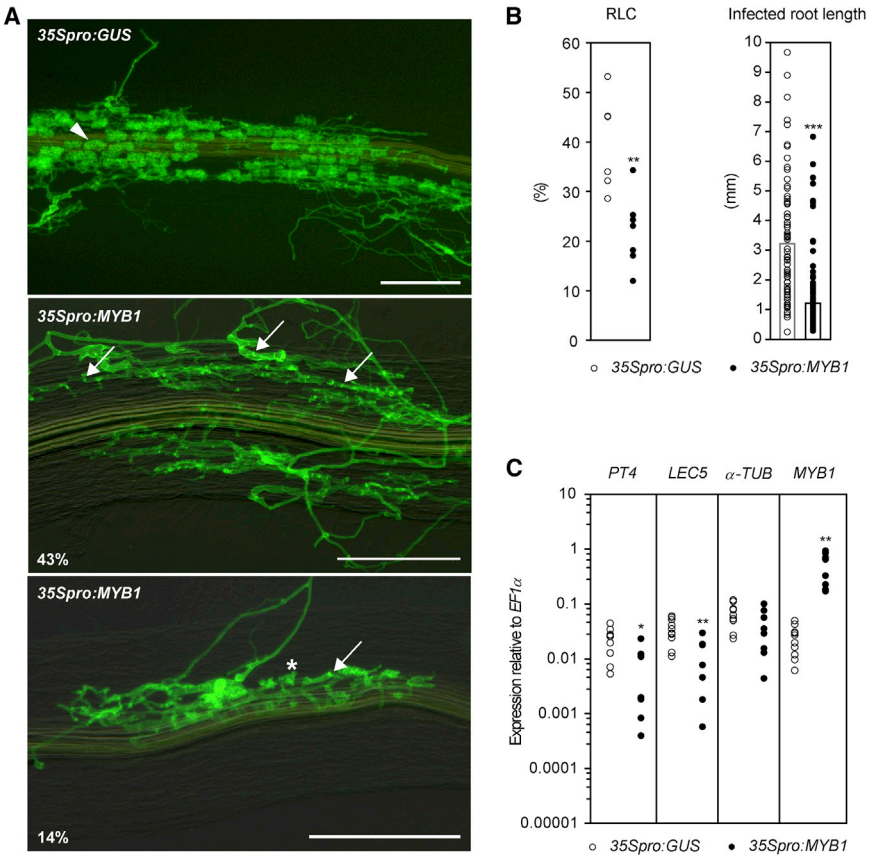

Figure 3. Constitutive Overexpression of *MYB1* Restricts Proliferation of *G. versiforme*

(A) Images of *G. versiforme* in *M. truncatula* roots expressing either a *35Spro*:*GUS* (vector control) or *35Spro*:*MYB1* at 30 dpp. An arrowhead marks a mature arbuscule. Arrows indicate septa. An asterisk marks a degenerated arbuscule. Percentages indicate the proportion of that particular AM phenotype in *35Spro*:*MYB1* roots (scale bars, 200 μm). Note, 43% of the infection units in *35Spro*:*MYB1* roots contained some large arbuscules as seen in the *35Spro*:*GUS* (vector control) roots.

(B) Colonization levels ($n \geq 6$ biological replicates) and infected root length of *35Spro*:*GUS* and *35Spro*:*MYB1* roots (data taken from between 9 and 28 randomly selected infections from six and seven biological replicate samples of *35Spro*:*GUS* [70 infections] and *35Spro*:*MYB1* roots [141 infections], respectively). $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; t test except for infected root length data, which show individual values and their averages (bars; statistical tests described in Supplemental Experimental Procedures).

(C) Expression of *PT4*, *LEC5*, *G. versiforme* α-*tubulin* (α-*TUB*), and *MYB1* in *35Spro*:*GUS* and *35Spro*:*MYB1* roots in an independent experiment at 28 dpp. $n \geq 7$ biological replicates. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; t test.

(Figure 3C). Thus, inappropriate expression of *MYB1* and presumably the hydrolase genes is detrimental to symbiosis. Despite the strong effect, 43% of infections showed occasional fully developed arbuscules, suggesting that overexpression of *MYB1* alone is not sufficient to fully induce arbuscule degeneration.

Transcription factors typically work in complexes, and therefore, we considered whether MYB1 might operate in concert with other factors known to regulate AM symbiosis. DELLA proteins are regulators of gibberellic acid signaling [36], and we and others have shown previously that they are positive regulators of arbuscule development [21, 22, 37, 38]. In a *della1 della2* double mutant, arbuscule numbers are very low [21], and this is true also of a *della1 della2 della3* triple mutant that lacks any functional DELLA proteins (Figures S4A–S4D). However, within these small populations of arbuscules, the proportion of large arbuscules, and therefore the mean arbuscule size (by length), is greater in the *della* double and *della* triple mutants relative to wild-type, whereas proportions of small arbuscules are reduced relative to wild-type (Figure 4A). These data suggest that, in addition to influencing arbuscule development, DELLAs may also influence arbuscule degeneration. Consequently, we examined the ability of MYB1 to promote expression of the hydrolase genes in a *della1 della2* mutant. In non-colonized *della1 della2* roots expressing *35Spro*:*MYB1*, transcript levels of degeneration-associated genes were significantly lower than in corresponding wild-type control roots (Figure 4B), which suggests that DELLAs and/or additional transcription factors that are regulated by DELLAs are involved in the MYB1-driven expression of degeneration-associated genes. As the expression analyses were carried out in non-colonized roots, the factors involved must be present prior to symbiosis. This point focused our attention to two constitutively expressed but symbiosis-associated GRAS factors, NSP1 and NSP2, whose expression is positively regulated by DELLAs [21, 35]. Also, arbuscule numbers are higher in an *nsp1 nsp2* double mutant relative to wild-type [39], which could result from an increase in arbuscule lifespan. To evaluate their potential role in regulating degeneration-associated gene expression, we expressed *MYB1* in an *nsp1 nsp2* double mutant (Figure S4E) and subsequently in the *nsp1* and *nsp2* single mutants (Figure 4C). These experiments revealed that NSP1, but not NSP2, is required for MYB1-induced expression of the *CP3*, *CP4*/*CP5*, *chitinase*, and *TGL* genes (Figure 4C). Although NSP1 is best known as a regulator of nodulation [5] and of strigolactone biosynthesis [39], *NSP1* is induced in colonized cortical cells during AM symbiosis, particularly at later stages of colonization [40]. Thus, overall, our data and the previous observations of *NSP1* expression are consistent with an additional function for NSP1 in arbuscule degeneration.

Given that expression of the degeneration-associated genes requires MYB1 and NSP1 and is promoted by DELLA, we evaluated the physical interactions between MYB1, NSP1, and DELLAs. In yeast two-hybrid analyses, MYB1 interacts with DELLAs and with NSP1 (Figure 4D). These interactions were confirmed in co-immunoprecipitation assays of transiently expressed proteins in *Nicotiana benthamiana*, where hemagglutinin (HA)-tagged MYB1 co-immunoprecipitated with yellow fluorescent protein (YFP)-tagged DELLA and MYC-tagged

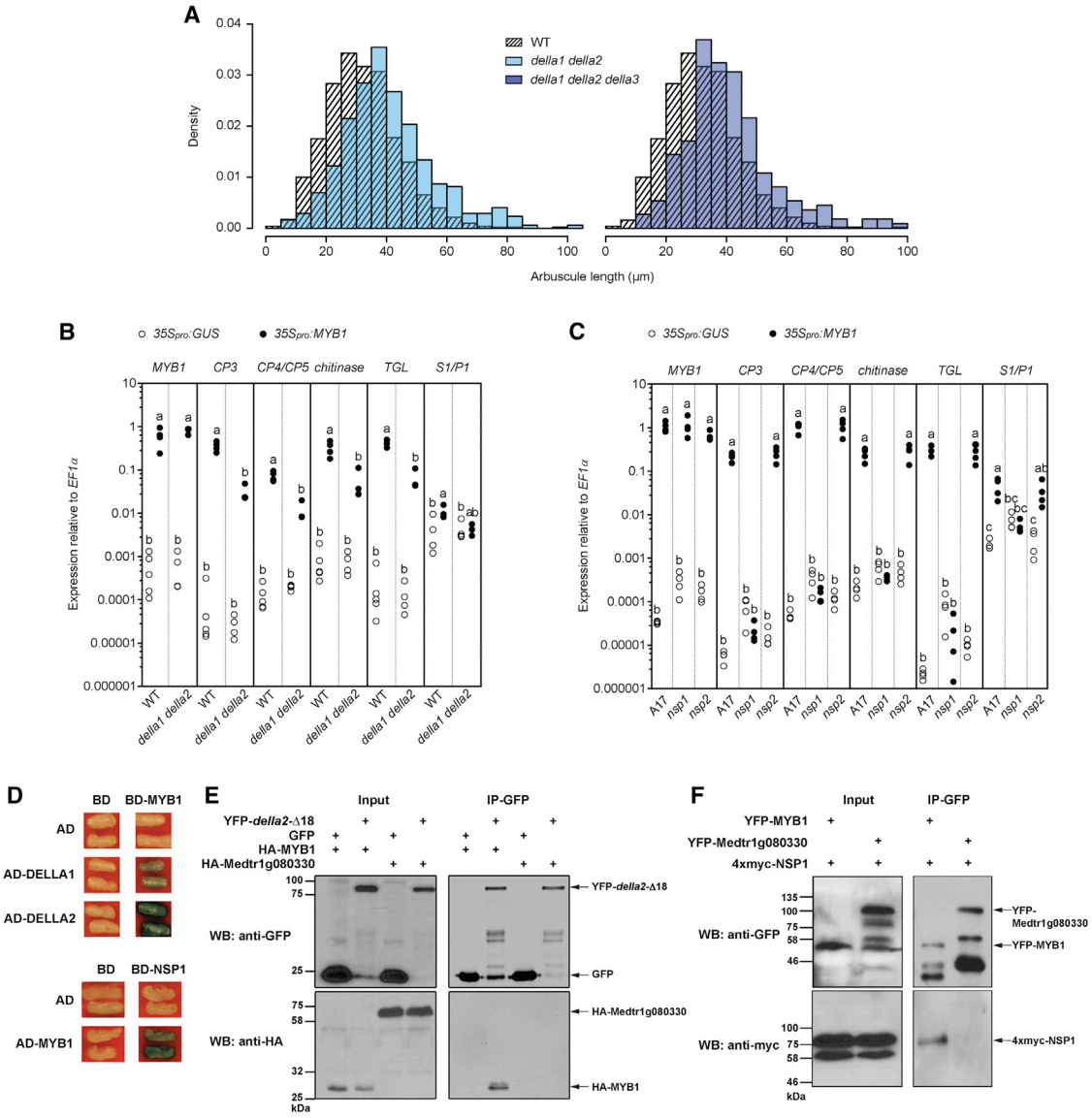

Figure 4. DELLA and NSP1 Transcriptional Regulators Are Required for MYB1-Induced Expression of Hydrolase Genes

(A) Distribution of arbuscules based on length in populations in WT, a *della1 della2* double mutant, and a *della1 della2 della3* triple mutant at 28 days post-inoculation with *G. versiforme*. Arbuscule length data are derived from measurements of wild-type (n = 1,003), *della1 della2* (n = 344), and *della1 della2 della3* (n = 222) arbuscules, and the histograms show arbuscule length distributions as probability densities to normalize for sample size. The arbuscule lengths in *della1 della2* (mean 40.6 ± 15.1 μm) and *della1 della2 della3* (mean 40.8 ± 14.8 μm) differ significantly from wild-type (mean 32.2 ± 12.0 μm; $p < 0.0001$; t test), but not from each other. Cortical cell size in the inner cortex region does not vary between the lines (WT, average cell length 45.3 ± 11.4 μm from n = 83 cells; *della1 della2*, average cell length 44.3 ± 14.3 μm from n = 100 cells; *della1 della2 della 3*, average cell length 45.6 ± 11.6 μm from n = 102 cells).

(B and C) Transcript levels of hydrolase genes in *della1 della2* roots (B) and *nsp1* and *nsp2* roots (C), and respective controls, expressing either a *35Spro*:*GUS* (vector control) or *35Spro*:*MYB1* at (B) 28 dpp and (C) 22 dpp. Data shown are the individual values of greater than or equal to three biological replicates. Statistical analysis was performed with ANOVA followed by Tukey HSD test. Different letters indicate significant differences ($p \leq 0.05$). Additional data are in Figure S4.

(D) Interaction between MYB1 and DELLA1, DELLA2, and NSP1 in a yeast two-hybrid assay. AD, LexA-activation domain; BD, LexA-binding domain.

(E and F) Co-immunoprecipitation assay showing MYB1 interaction with (E) DELLA2 and (F) NSP1 in *N. benthamiana* leaves. To increase sensitivity, a gibberellic acid (GA)-insensitive mutant protein of DELLA2 (*della2*-Δ18) was used. Medtr1g080330 tagged either with HA or YFP serves as a negative control. Additional data are in Figure S4.

NSP1 co-immunoprecipitated with YFP-tagged MYB1. A second member of the MYB family (Medtr1g080330; Figure S2E) was used as a negative control and did not co-immunoprecipitate with YFP-tagged DELLA and was unable to co-immunoprecipitate MYC-tagged NSP1 (Figures 4E and 4F). The finding that both DELLA and NSP1 interact with MYB1 supports a direct role for DELLA in promoting expression of degeneration-associated genes rather than simply an indirect role through influences on

NSP1 expression. Taken together with the mutant analyses and hydrolase gene expression, the interaction data suggest that MYB1 operates with both DELLA and NSP1 to regulate expression of the arbuscule degeneration-associated hydrolases.

In summary, during AM symbiosis, the root cortical cell is challenged first with the construction of the PAM and matrix in which to temporarily house its fungal symbiont and then with the subsequent disassembly of these components following arbuscule degeneration. The transcriptional reprogramming associated with development of the interface has been well documented [27, 28, 34]. Here, we identify a distinct set of AM-inducible genes associated with arbuscule degeneration that is dominated by secreted hydrolases likely involved in interface disassembly. Expression of these degeneration-associated genes is regulated by MYB1, an AM-inducible transcription factor. Surprisingly, *MYB1* transcripts increase rapidly during symbiosis and parallel those of *PT4*, yet as illustrated in the *MYB1* overexpression experiments, inappropriate expression of *MYB1* impairs symbiotic development. Consequently, we predict that post-translational regulation of MYB1 activity prevents it from acting prematurely. There is a precedent for this; in *Arabidopsis* and rice, regulation of MYB transcription factors involved in phosphate-starvation signaling occurs through interactions with SPX proteins [41, 42].

Although MYB1 drives expression of degeneration-associated genes, this function is dependent on a constitutively expressed GRAS transcription factor NSP1 and enhanced by a constitutively expressed DELLA protein. Both NSP1 and DELLA also act at earlier stages of the symbiosis and function in transcription factor complexes [20, 38, 43] to control the biosynthesis of initial signaling molecules [39] and in arbuscule development [21, 22, 38]. These data, coupled with the identification of physical interactions of MYB1 with NSP1 and with DELLA, suggest that assembly of new transcription factor complexes, comprised partially of factors that also regulate symbiotic development, enables the cell to achieve sequential expression of the development and degeneration programs. Furthermore, the involvement of DELLAs, whose activities are influenced by nutritional status and NSP1, which regulates nodulation [5], provides avenues for cross-talk with other signaling pathways.

ACCESSION NUMBERS

The NCBI Gene Expression Omnibus accession number for the microarray data reported in this paper is GEO: GSE95545.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.03.003.

AUTHOR CONTRIBUTIONS

D.S.F. contributed the majority of the data except for the following: S.K.G. contributed the GeneChip data, H.-J.P. constructed the yeast two-hybrid library and contributed yeast DELLA/MYB interaction data and *ram1* data, A.M.M. contributed MYB1/NSP1 coIP data, L.M.M. contributed analysis of the *della* triple mutant, K.K.B. contributed qPCR validation of the GeneChip data and the initial CP3 promoter-GUS data, V.L.-T. contributed qPCR data, and I.E.M.-M. and M.J.H. contributed the initial MYB RNAi data. M.J.H. and D.S.F. wrote the manuscript.

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