DELLA proteins regulate arbuscule formation in arbuscular mycorrhizal symbiosis

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Most flowering plants are able to form endosymbioses with arbuscular mycorrhizal fungi. In this mutualistic association, the fungus colonizes the root cortex and establishes elaborately branched hyphae, called arbuscules, within the cortical cells. Arbuscule development requires the cellular reorganization of both symbionts, and the resulting symbiotic interface functions in nutrient exchange. A plant symbiosis signaling pathway controls the development of the symbiosis. Several components of the pathway have been identified, but transcriptional regulators that control downstream pathways for arbuscule formation are still unknown. Here we show that DELLA proteins, which are repressors of gibberellic acid (GA) signaling and function at the nexus of several signaling pathways, are required for arbuscule formation. Arbuscule formation is severely impaired in a *Medicago truncatula Mtdella1/Mtdella2* double mutant; GA treatment of wild-type roots phenocopies the *della* double mutant, and a dominant DELLA protein (*della1*-Δ18) enables arbuscule formation in the presence of GA. Ectopic expression of *della1*-Δ18 suggests that DELLA activity in the vascular tissue and endodermis is sufficient to enable arbuscule formation in the inner cortical cells. In addition, expression of *della1*-Δ18 restores arbuscule formation in the symbiosis signaling pathway mutant *cyclops/ipd3*, indicating an intersection between DELLA and symbiosis signaling for arbuscule formation. GA signaling also influences arbuscule formation in monocots, and a Green Revolution wheat variety carrying dominant DELLA alleles shows enhanced colonization but a limited growth response to arbuscular mycorrhizal symbiosis.

endosymbiosis | phytohormone | biotrophic | *cyclops* | *Lotus japonicus*

Arbuscular mycorrhizal (AM) symbiosis, formed by most angiosperms and fungi of the order Glomeromycota (1, 2), occurs widely in terrestrial ecosystems, where it plays a significant role in plant phosphorus nutrition and the carbon cycle and consequently impacts ecosystem productivity (1, 3). Given the limitations of global phosphate rock reserves, sustainable use of Pi fertilizer in agriculture will become increasingly important, and AM symbiosis may contribute to this sustainability (4).

To initiate AM symbiosis, root cells, primed by signals from the fungus, activate a symbiosis signaling pathway (SSP), which triggers cellular rearrangements that permit growth of the fungal hyphae through the epidermal cells and into the cortex. A second phase of differentiation results in hyphal growth into cortical cells and development of branched hyphae called arbuscules (reviewed in refs. 5 and 6). Phosphorus and nitrogen are delivered to the root through arbuscules, and in return the fungus gains access to carbon (7–10). The SSP was first identified in legumes, where it also functions in symbiosis with nitrogen-fixing rhizobia (11). Several components of the pathway have been identified, and in SSP mutants AM symbiosis is blocked at different stages of development. In three SSP mutants, including those with a mutation in a calcium calmodulin-dependent protein kinase (CCAMK/DMI3), hyphal growth is arrested in the epidermis (12–15). CYCLOPS/IPD3, a protein of unknown function that interacts with CCAMK, influences cortical colonization, and in *Lotus japonicus* and rice *cyclops* mutants, AM fungal hyphae grow into the cortex, but arbuscules are not formed (14, 16). The phenotypes of some *Medicago truncatula ipd3* alleles are similar to those of *cyclops*, but arbuscules are formed in others (17–19). VAPYRIN/PAM2, also of unknown function, likewise is required for arbuscule formation, although currently it is unclear whether this protein is a signaling protein (20–22). Our current understanding of the SSP and how it functions to regulate AM symbiosis is incomplete. In rhizobium legume symbiosis (RLS), signaling through the SSP ultimately activates transcription factors that control infection and nodule development (23). A similar model is proposed for AM symbiosis, and GRAS factors that regulate hyphopodia development (24) and cortical colonization levels have been identified (25), but regulators specific for arbuscule formation are unknown. Transcript profiling and promoter–reporter gene analyses indicate complex changes in plant gene expression in the root cortex during arbuscule development, suggesting that multiple signaling pathways may be involved in arbuscule formation (14, 26–30). Complex regulation of arbuscule formation might be expected, because the symbiosis is modulated in response to the plant's phosphate status, nitrogen status, and photosynthetic capacity (31–34). It is not known if regulation in response to the plant's physiological status involves the SSP.

Transcriptome analyses reveal substantial alterations in the expression of genes encoding enzymes of gibberellic acid (GA) biosynthesis, degradation, and signaling during AM symbiosis (28, 35–39). Consistent with these alterations, GA levels increase significantly in mycorrhizal roots (40). Furthermore, researchers studying polyamine biosynthesis in mycorrhizal roots observed

Significance

Arbuscular mycorrhizal (AM) symbiosis is a mutualistic interaction formed between most land plants and soil fungi. During symbiosis the fungus develops branched hyphae, known as arbuscules, inside the root cortical cells. Arbuscules are critical to the symbiosis and function in phosphate delivery to the plant. Here we show that arbuscule formation is regulated by DELLA proteins. DELLA proteins are negative regulators of gibberellic acid (GA) signaling and repress plant growth and development. Our data provide insights into regulation of arbuscule formation and identify a potential mechanism by which the plant can coordinate the symbiosis with its growth and nutrient status.

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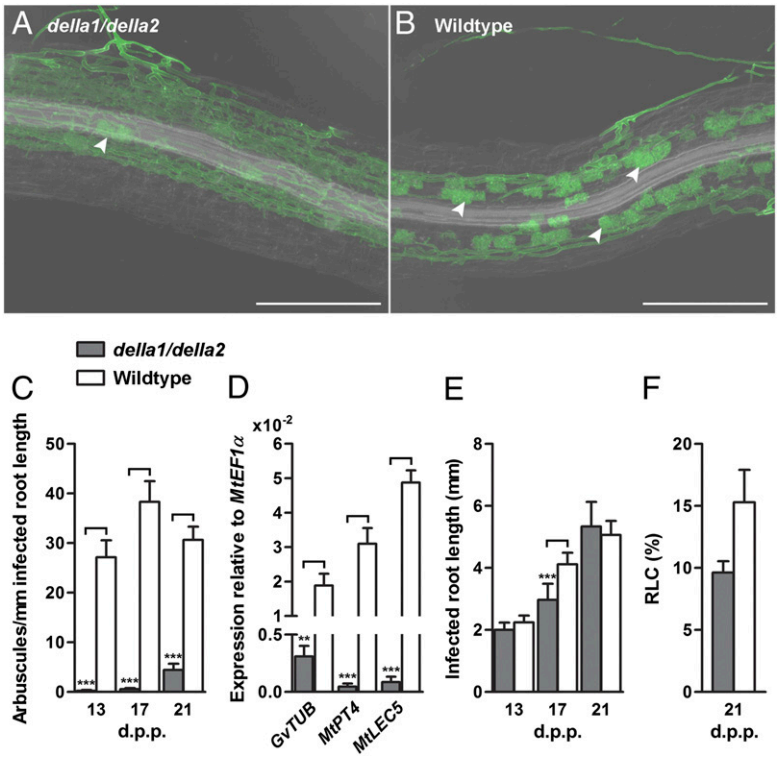

Fig. 1. AM phenotype of *della1/della2* roots colonized by *G. versiforme*. (*A* and *B*) Laser-scanning confocal microscope images of *G. versiforme* in *della1/della2* (*A*) and wild-type (*B*) roots. Arrowheads mark arbuscules. (Scale bars: 250 μm.) (*C*) Arbuscule density in *della1/della2* and wild-type roots at 13, 17, and 21 d post planting (d.p.p.). Data are averages ± SEM ($n \geq 24$, where *N* denotes the number of infected root sections). (*D*) Expression of *G. versiforme* α-*tubulin* (*GvTUB*), *MtPT4*, and *MtLEC5* in roots 21 d post inoculation (d.p.i.) assayed by quantitative RT-PCR. Data are averages ± SEM ($n \geq 5$, where *N* denotes the number of independent root samples). (*E*) Infected root length in *della1/della2* roots does not differ dramatically from that in wild-type roots, although significant changes are seen at 17 d.p.p. Data are averages ± SEM ($n \geq 24$, where *N* denotes the number of infected root sections). (*F*) Level of colonization of *della1/della2* and wild-type roots. Data are averages ± SEM ($n = 3$, where *N* denotes the number of independent root samples). RLC, root length colonized. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

that GA treatment inhibited the development of AM symbiosis and in particular arbuscule number (41).

GA is a phytohormone that controls many aspects of plant growth and development and also influences responses to abiotic and biotic stress (42–47). GAs are synthesized from carotenoid precursors, and bioactive GA levels are modulated by a combination of GA synthesis and GA degradation (48, 49). Through studies in *Arabidopsis* and rice, a mechanistic understanding of GA perception and signaling has been obtained (reviewed in refs. 50–52). DELLA proteins, a unique group of GRAS transcriptional regulators, are central players in GA signaling and repress GA responses and restrain growth (53, 54). There are five DELLA proteins in *Arabidopsis*, two in pea, and one in rice (55–61). DELLA proteins contain domains typical of other GRAS transcription factors (62), but in addition they contain a unique DELLA domain at the N terminus. In the presence of GA, the DELLA domain mediates interaction with the GID1 receptor, and DELLA proteins subsequently are degraded via the 26S proteasome (51–54). Removal of DELLA proteins thus relieves repression and enables growth and other GA-activated responses (53, 54, 63). DELLA proteins interact physically with many transcriptional regulators in diverse signaling pathways and interfere with or modulate transcription factor function (64–70). As a consequence, GA has wide-reaching effects on gene expression, and DELLA proteins mediate cross-talk between many signaling pathways. In *Arabidopsis*, a role for DELLA proteins and GA signaling in Pi-starvation signaling has been established, and DELLA proteins regulate a subset of the adaptive responses to Pi starvation, including alterations in root architecture (71).

Here, we demonstrate that in *M. truncatula* DELLA1 and DELLA2 are required for arbuscule formation, as is consistent with a recent report that arbuscule formation is impaired in a pea *cry, la* mutant (72). Surprisingly, in *M. truncatula della1/della2* mutants, hyphal growth in the cortex is not impaired by the lack of arbuscules, suggesting that intercellular hyphae have the capacity to access carbon. A dominant DELLA mutant restores arbuscule formation in *cyclops*, indicating an intersection between DELLA signaling and the SSP. Thus, DELLA proteins provide a mechanism for connecting symbiosis signaling with plant growth and development.

Results

***M. truncatula DELLA1* and *DELLA2* Are Required for Arbuscule Formation.** A reverse genetic screen that aimed to identify *M. truncatula* genes involved in AM symbiosis revealed that RNAi knockdown of a DELLA gene resulted in aberrant AM symbiosis. In DELLA RNAi roots inoculated with *Glomus versiforme*, hyphal growth into the roots occurred as in wild-type roots, but development in the cortex was altered, and arbuscule formation was markedly reduced (Fig. S1). There are three *DELLA* genes in the *M. truncatula* genome database Mt3.5: MtDELLA1 (contig_170694 and contig_69957), MtDELLA2 (contig_52215), and MtDELLA3 (contig_55897). The encoded proteins share 56–68% identity with DELLA proteins of *Arabidopsis* (Fig. S2), and *MtDELLA1* and *MtDELLA2* are orthologs of Pea LA and CRY (60), which recently were shown to influence arbuscule formation (72). To confirm and extend the RNAi results, *M. truncatula* lines containing *Tnt1* insertions in *MtDELLA1* and *MtDELLA2* were obtained from a mutant population generated at the Samuel Roberts Noble Foundation. We

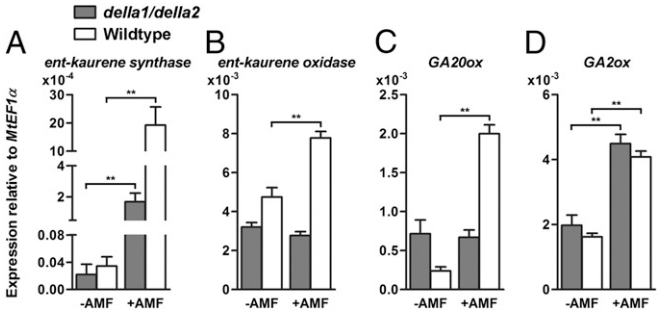

Fig. 2. Transcript levels of genes encoding enzymes of GA biosynthesis in noncolonized (–AMF) *della1/della2* and wild-type roots and in *della1/della2* and wild-type roots colonized with *G. versiforme* (+AMF) at 21 d.p.i. as assayed by quantitative RT-PCR. GAs are synthesized from geranylgeranyl diphosphate (GGDD) (116). (*A* and *B*) *ent*-kaurene synthase is involved in the conversion of GGDD to *ent*-kaurene, which is further converted to *ent*-kaurenoic acid, catalyzed by *ent*-kaurene oxidase. *Ent*-kaurenoic acid is subsequently converted to GA_{12}. (*C*) GA 20-oxidases (GA20ox) catalyze the sequential oxidation of C-20 to convert GA_{12} to bioactive GAs. (*D*) GA 2-oxidases (GA2ox) catalyze GA inactivation reactions. Data are averages ± SEM ($n \geq 6$, where *N* denotes the number of independent roots samples). $^{**}P \leq 0.01$.

focused on these two *DELLA* genes because they are coexpressed and because their transcript levels are high in *M. truncatula* Pi-deprived roots and remain high during AM symbiosis (Fig. S3 *A* and *B*). Expression of both genes is reduced after the addition of phosphate (Fig. S3 *C* and *D*). Two lines with insertions in *MtDELLA1* and one line with an insertion in *MtDELLA2* were obtained (Fig. S3*E*) and crossed to create a *della1/della2* double mutant. The *della1/della2* mutant showed developmental phenotypes characteristic of *Arabidopsis* and rice *della* mutants, namely, a slender shoot (61), early flowering, and reduced root fresh weight (Fig. S4). After inoculation with *G. versiforme*, *della1* and *della2* single mutants each showed an AM phenotype that did not differ from that of the wild-type segregant controls; however, the AM phenotype of the *della1/della2* double mutant largely recapitulated the phenotype of the RNAi roots, and arbuscule formation was drastically reduced relative to the controls (Fig. 1 *A*–C and Fig. S5). On average, the arbuscule density in *della1/della2* mycorrhizal roots was 85% lower than in control roots. The AM symbiosis phenotype of the *della1/della2* mutant was examined in a range of substrates and nutritional conditions, and arbuscules were entirely absent in some experiments (Fig. S5). *MtPT4* and *MtLEC5* transcripts, which are markers of cells containing arbuscules (73–75), were extremely low in *della1/della2* roots, and *G. versiforme α-tubulin* transcripts, which provide an indication of fungal biomass, likewise are lower in *della1/della2* mutant than in wild-type roots (Fig. 1*D*). In *Arabidopsis*, several GA biosynthesis genes, including *GA20ox2*, are direct targets of DELLA proteins (76). In *M. truncatula*, genes encoding enzymes of GA biosynthesis, which are highly induced in wild-type roots during symbiosis, either are not induced or are induced only marginally in *della1/della2* roots (Fig. 2 *A*–*C*). Expression of GA-2-oxidase, an enzyme that degrades bioactive GA, is similar in wild type and *della1/della2* mutants (Fig. 2*D*). Despite the lack of arbuscules in *della1/della2* mutants, intercellular hyphal growth in the cortex was not impaired, and infected root lengths in the double mutant did not differ significantly from those of wild-type roots (Fig. 1 *E* and *F* and Fig. S5*C*). The extensive growth of intercellular hyphae in *della1/della2* roots suggests that the fungus is able to obtain carbon in the absence of arbuscules. However, the fungus fails to produce new spores and thus cannot complete its life cycle in *della1/della2* roots (Table 1).

A Dominant DELLA Mutant Protein Promotes Arbuscule Formation. DELLA proteins are negative regulators of GA signaling, and their repressive function is relieved by GA-induced proteolytic degradation (52, 54, 77, 78). Thus application of GA to mycorrhizal roots would be expected to phenocopy the *della1/della2* mutant, and a dominant *della* mutant that is insensitive to GA but maintains function (55) would be expected to enable arbuscule formation even in the presence of GA. It has been shown previously that GA treatment suppresses arbuscule development in pea (41, 72), although in these experiments the effects on the fungus and plant could not be easily distinguished. The application of GA_3 to *M. truncatula* roots inoculated with *G. versiforme* resulted in the expected alterations in plant growth and had a significant impact on development of symbiosis. *G. versiforme* was able to develop intercellular hyphae in the cortex, but arbuscule development was abolished completely (Fig. 3 *A* and *B*). As observed in *della1/della2* mycorrhizal roots, intercellular hyphal growth in the cortex was unimpaired, and infection unit lengths in the GA-treated roots did not differ from those in control roots (Fig. 3*C*).

In *Arabidopsis*, the semidominant *gai-1* allele encodes a mutant DELLA protein that lacks the DELLA domain; the repressor function is unimpaired, but the protein is insensitive to GA (55). Guided by this information, we created a dominant version of MtDELLA1 (*della1*-Δ18) by deleting the DELLA domain, and this version was introduced into wild-type roots under the control of the 35S promoter. After inoculation and GA_3 treatment, the vector control roots showed a typical GA-treated AM phenotype with intercellular hyphae and no arbuscules. In contrast, arbuscule formation was restored in roots expressing *della1*-Δ18, with 40–50% of the infections showing arbuscules (Fig. 3 *D* and *E*). Likewise, arbuscule formation was restored in *della1/della2* mutants expressing *MtDELLA1* promoter:*della1*-Δ18 (Fig. S6 *A* and *B*). In both cases, arbuscules resulting from *della1*-Δ18 expression showed a normal morphology (Fig. S6*C*). Taken together, the phenotypes of the *della1/della2* mutant and the effects of the *della1*-Δ18 constructs indicate that GA signaling through DELLA proteins regulates arbuscule formation.

Expression of *della1*-Δ18 in the Vascular Tissue and Endodermis Enables Arbuscule Formation in the Inner Cortical Cells. In the AM symbiosis formed between *M. truncatula* and *G. versiforme*, arbuscules develop in the inner cortical cell layers. However, based on analysis of an *MtDELLA1* promoter:GUS construct, *MtDELLA1* is expressed strongly in the vascular tissue and endodermis but is detected in inner cortical cells only in rare instances and then only at extremely low levels (Fig. 4*A*). The vascular/endodermal expression pattern of *MtDELLA1* promoter:GUS is maintained after colonization, and GUS expression was not observed in cells with arbuscules (Fig. 4*B*). These data are consistent with *MtDELLA1* transcript levels, which do not change significantly after colonization (Fig. S3*B*). To monitor the DELLA protein, we created an *MtDELLA1* promoter:GFP-MtDELLA1 fusion but were unable to detect its expression in *M. truncatula* roots. To increase the sensitivity, a GFP fusion to the GA-insensitive *della1*-Δ18 mutant protein was created; this construct complemented the arbuscule defect of the *della1/della2* mutant, indicating that the fusion protein was functional (Fig. S6 *A* and *B*). When expressed from the *MtDELLA1* promoter, GFP- *della1*-Δ18 was visible in the nuclei of cells in the

Table 1. *G. versiforme* spore production during association with *della1/della2*

Genotype	Spores per 1-cm^2 area
della1/della2	0 (n = 65 sections)
Wild type	10 ± 2 (n = 56 sections)

Spores were counted in 1-cm^2 areas across the root system.

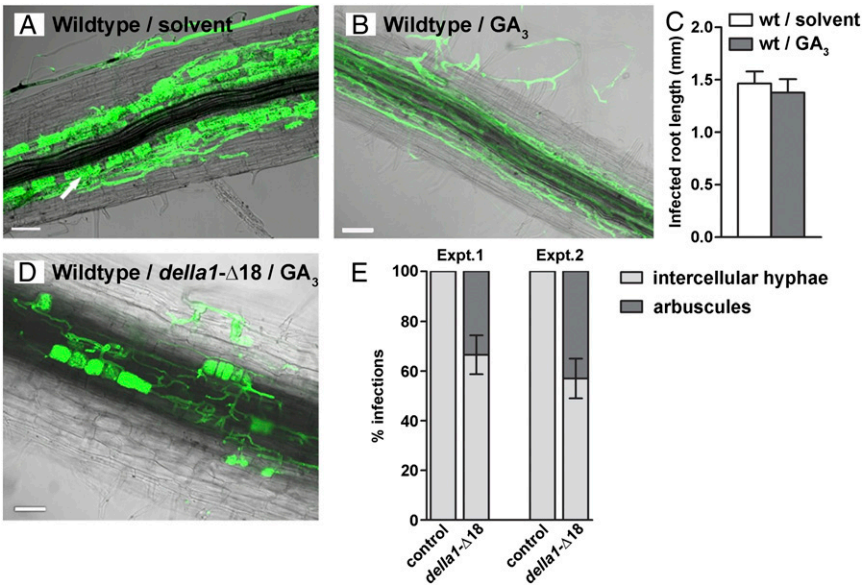

Fig. 3. AM phenotype of *M. truncatula* roots and transgenic roots expressing a dominant DELLA1 gene (*della1*-Δ18) after GA_3 treatment. (*A* and *B*) Fluorescence/differential interference contrast overlay images of *G. versiforme* in roots of *M. truncatula* after treatment with solvent (*A*) or GA_3 (*B*). The arrow in *A* marks an arbuscule. Arbuscules are absent in GA_3-treated roots. (*C*) Infected root length in GA_3-treated roots does not differ from control. Data are averages ± SEM ($n \geq 31$). (*D*) Morphology of *G. versiforme* in roots expressing *della1*-Δ18 after treatment with GA_3. Arbuscule formation is restored. (*E*) Percentage of infected root sections containing only intercellular hyphae or arbuscules after GA_3 treatment in roots expressing a vector control or *della1*-Δ18. Data are averages ± SEM from two independent experiments ($n \geq 10$). (Scale bars: 50 μm.)

vascular tissue and endodermis (Fig. S6*D*) and also, sporadically, in some of the inner cortical cells (Fig. 4 *C* and *D* and Fig. S6 *E* and *F*).

Because *MtDELLA1* promoter activity was detected consistently in the vascular tissue/endodermis and was sporadic or almost absent in the cortex, we questioned whether DELLA activity in the vascular tissue/endodermis was sufficient to enable arbuscule formation. In *Arabidopsis*, DELLA activity in the endodermal layer plays a major role in the control of root elongation, and the endodermis is particularly sensitive to expression of *gai,* a dominant DELLA protein (79). To investigate further the potential site of DELLA action, the *della1*-Δ18 gene was placed under the control of the *MtPT9* promoter. *MtPT9* (Medtr4g083960) encodes a phosphate transporter of the Pht1 family and is expressed in roots and shoots and during AM symbiosis in both wild type and *della1*/*della2* mutants (Fig. S7 *A*, *B*, and *E*). The *MtPT9* promoter is highly active in the vascular tissue and endodermis (Fig. S7 *C*, *D*, and *F*). Expression of *pMtPT9:della1*-Δ18 restored arbuscule formation in the inner cortical cells of *della1*/*della2* roots (Fig. 4 *E* and *F*) and also in wild-type roots treated with GA. These data indicate that expression of DELLA in the vascular tissue and endodermis is sufficient to drive arbuscule formation in the inner cortex cells. Consequently, we suggest that, when expressed from the native promoter, DELLA activity in these cell types may control arbuscule formation, although an additional contribution from the cortex cannot be ruled out.

DELLA Proteins and the Symbiosis Signaling Pathway. Development of AM symbiosis is controlled by the SSP; however, current data indicate that activation of the SSP pathway alone is not sufficient to induce the complete cortical transcriptional program associated with arbuscule formation (80) and that other pathways may be involved. To test the hypothesis that DELLA signaling for arbuscule formation might intersect with the SSP, the *della1*-Δ18 gene was expressed from the 35S promoter in a *L. japonicus cyclops* mutant, a *M. truncatula ipd3* mutant, and in two additional symbiosis mutants, *M. truncatula dmi3* and *M. truncatula vapyrin,* that are positioned upstream and downstream of *cyclops*/*ipd3* in the SSP, respectively. In *dmi3* mutants hyphal growth is arrested at the epidermis (12), whereas in *cyclops* mutants and some *ipd3* mutants the hyphae grow into the outer cortex, but arbuscules are absent (16, 18). In *vapyrin* mutants, hyphae reach the inner cortex, but arbuscules are not formed (20, 21).

Expression of *p35S:della1*-Δ18 did not affect the AM symbiosis phenotype of *dmi3* or *vapyrin*; however it altered the *L. japonicus cyclops* and *M. truncatula ipd3* phenotypes. After inoculation with *G. versiforme*, *L. japonicus cyclops* roots expressing *p35S:della1*-Δ18 showed arbuscules in 14% of the infections, but arbuscules were absent in *cyclops* roots expressing a control construct (Fig. 5 *A*–*C*). Expression of *p35S:della1*-Δ18 also increased intraradical hyphal growth in the cortex of *cyclops* roots by an average of 3.3-fold (Fig. 5*C*). The effect was not as obvious in *M. truncatula ipd3* roots because, although *ipd3-2* is reported to lack arbuscules (18), arbuscules were present in both *ipd3-1* and *ipd3-2* in our experimental conditions, although overall colonization levels were lower than in the wild-type controls (Fig. S8 *A* and *B*). However, in *ipd3-1* roots expressing *p35S:della1*-Δ18 there was a small, but statistically significant increase in arbuscule numbers (Fig. S8*C*) and in the overall level of colonization (Fig. S8*D*). Thus, repression of GA signaling via the expression of *della1*-Δ18 promotes arbuscule formation and increases hyphal development in the absence of CYCLOPS/IPD3.

The *L. japonicus cyclops* AM symbiosis phenotype is similar to that of the *della1*/*della2* mutant in that it lacks arbuscules (17) (Fig. 5 *D* and *E*); however *DELLA* and *GA-20-oxidase* transcript levels in *cyclops* roots do not differ significantly from those in wild-type roots, suggesting that the *cyclops* phenotype does not arise from a reduction in *DELLA* transcripts or from an increase in GA biosynthetic gene expression (Fig. 5 *F* and *G*). *NSP1* and *NSP2* transcript levels likewise are similar in *cyclops* and wild-type roots, although *cyclops* roots do not show increased *NSP1* transcripts following colonization (Fig. 5 *H* and *I*).

To evaluate DELLA and the SSP further, we analyzed transcript levels of the symbiosis-signaling genes *DMI3* and *IPD3* and two GRAS transcription factors, NSP1 and NSP2, in *della1*/*della2* mutants. There were no changes in *DMI3* and *IPD3* transcript

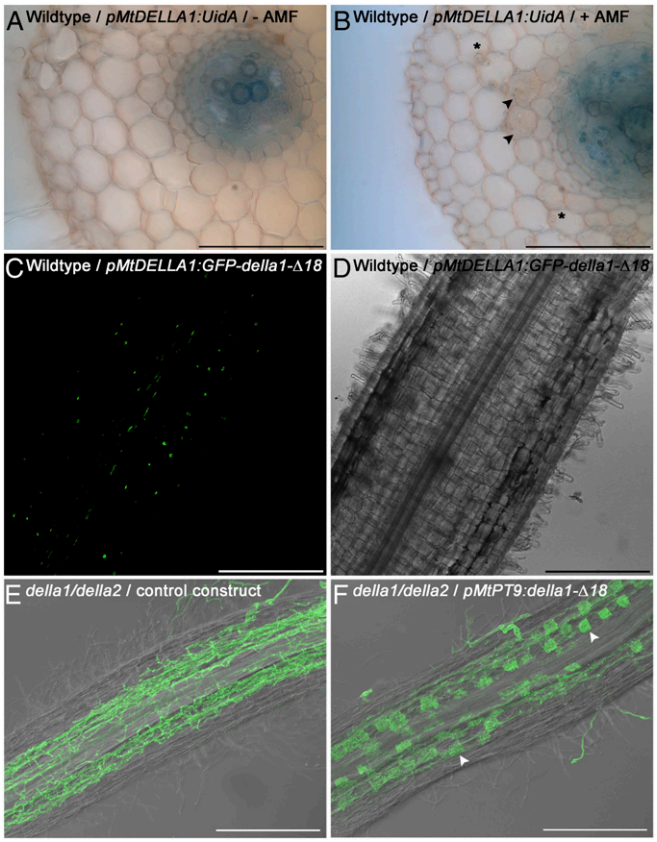

Fig. 4. Expression and localization of MtDELLA1 in *M. truncatula* roots and AM phenotype of *della1/della2* expressing *della1*-Δ18 driven by a vascular tissue-specific promoter. (*A* and *B*) Localization of *MtDELLA1* promoter activity in noncolonized roots (–AMF) (*A*) and in roots colonized by *G. versiforme* (+AMF) (*B*). GUS-stained roots expressing *pMtDELLA1:UidA* indicate promoter activity in the vascular tissue and endodermis. Arrowheads mark cells containing arbuscules. Stars mark fungal hyphae. (*C* and *D*) Roots expressing *pMtDELLA1:GFP-della1*-Δ18 show consistent GFP signals in the nuclei of cells of the vascular tissue and endodermis; weaker signals are seen in some, but not all, cortical cells. Note that in this image the vascular tissue is not entirely in the focal plane (see also Fig. S6*D*). (*E* and *F*) Laser-scanning confocal microscope images of *G. versiforme* in roots of the *della1/della2* mutant transformed with a control construct (*E*) or *pMtPT9:della1*-Δ18 (*F*). Expression of *pMtPT9:della1*-Δ18 restores arbuscule formation. (Scale bars: 100 μm in *A* and *B*; 250 μm in *C*–*F*.)

levels in *della1/della2* roots relative to the wild-type segregant controls (Fig. 6 *A* and *B*). However, *NSP1* and *NSP2* transcript levels were significantly lower in the *della1/della2* mutant than in the corresponding wild-type segregant, particularly during AM symbiosis (Fig. 6 *C* and *D*), suggesting that DELLA regulates *NSP1* and *NSP2* transcript levels either directly or indirectly. A link between GA and NSP2 has been reported during nodulation when Nod factor-induced expression of NSP2 was suppressed by GA treatment (81). Consistent with these data, we found that nodulation in the *della1/della2* mutant was reduced significantly (Fig. S9). NSP1 and NSP2 are GRAS factors that are positioned downstream of DMI3 and CYCLOPS/IPD3 and are required for nodulation (11). In addition, recent data suggest that both transcription factors influence AM symbiosis in a quantitative manner but do not influence the morphology of the symbiosis (25, 82, 83). As reported previously (84), we found that an *nsp1/nsp2* double mutant showed lower overall colonization levels relative to wild type (Fig. S10*A*). Expression of *della1*-Δ18 in *nsp1/nsp2* roots resulted in increased overall colonization levels and arbuscule formation, suggesting that these GRAS factors are not essential for the *della1*-Δ18–driven changes in arbuscule formation or intercellular hyphal development (Fig. S10 *B* and *C*). Consequently, we predict that DELLA proteins influence other, as yet unknown, transcription factors to control arbuscule formation.

AM Symbiosis in Green Revolution Crop Varieties. Wheat, maize, and rice are major food sources for the world's population, and several of the Green Revolution cereal crop varieties are dominant DELLA mutants (85). For example, most wheat varieties grown worldwide are high-yielding dwarf varieties that express *Reduced height* (*Rht*) alleles which encode dominant DELLA proteins (86). To determine whether breeding for short stature and high yield has inadvertently resulted in plants with altered responses to AM symbiosis, wheat lines carrying the Rht_1/Rht_2 dominant DELLA alleles or the rht_1/rht_2 wild-type alleles (87–89) were inoculated with *G. versiforme*, and colonization and growth were monitored. The wheat line expressing Rht_1/Rht_2 *alleles* showed a small but significant increase in colonization relative to the line expressing rht_1/rht_2 wild-type alleles (Fig. 7*A*), and *G. versiforme a-tubulin* transcript levels were significantly higher in the Rht_1/Rht_2 line (Fig. 7*B*). Furthermore, *TaPT10* and *TaPT11* transcripts, which encode AM-induced phosphate transporters belonging to the MtPT4 subfamily (90), also were significantly higher in the Rht_1/Rht_2 line than in the wild type (Fig. 7 *C* and *D*), suggesting an increase in arbuscules numbers. These data are consistent with the effects observed in *M. truncatula* expressing *della1*-Δ18, and we observed a similar effect in Maize D8 (85, 91), a dominant *della* mutant. *DXR* transcripts, which serve as markers of arbuscule-containing cells (92), were higher in D8 than in the wild-type control (Fig. S11). In the wheat Rht_1/Rht_2 line, the arbuscules appear to be functional, because this line shows a significant increase in shoot phosphorus content during symbiosis (Fig. 7*E*). Despite the high colonization levels and the increase in shoot phosphorus content, the Rht_1/Rht_2 line did not show a symbiosis-associated increase in shoot biomass (Fig. 7*F*), likely because the dominant DELLA proteins restrain shoot growth. In conclusion, under the growth conditions tested, expression of the dominant DELLA Rht1/Rht2 proteins does not greatly impact the functionality of AM symbiosis in wheat.

Discussion

The AM symbiosis is of central importance to plant mineral nutrition, and arbuscules are critical for nutrient exchange between the fungal and plant symbionts. Arbuscule development is controlled in part by the plant; however, the regulatory mechanisms are largely unknown (reviewed in ref. 5). Here we show that two DELLA proteins, MtDELLA1 and MtDELLA2, are required for arbuscule formation. Treatment of *M. truncatula* roots with GA results in a phenocopy of *della1/della2*, and arbuscules can be restored in GA-treated roots by the expression of a GA-insensitive *della1*-Δ18 mutant protein. Taken together, these data confirm a role for GA as a negative regulator of arbuscule formation. In mycorrhizal roots, it is possible that the site of DELLA action may not be directly in the cortical cells where arbuscules develop, because ectopic expression of *della1*-Δ18 specifically in the vascular tissue and endodermis enables arbuscule formation in GA-treated roots and also in the *della1/della2* mutant. These experiments establish that DELLA can act from the vascular tissue and endodermis, but in the native situation a contribution from DELLA in the cortex cannot be ruled out. If the site of action is the vascular tissue and endodermis, DELLA proteins may interact with transcription factors that subsequently move to the cortex; alternatively, DELLA regulation of arbuscule formation may be indirect. DELLA proteins influence several other phytohormone-signaling pathways, so responses in the cortex could result from changes in other mobile signaling molecules (66, 69); alternatively, a temporary restraint of root growth (79) may be necessary to enable arbuscule formation.

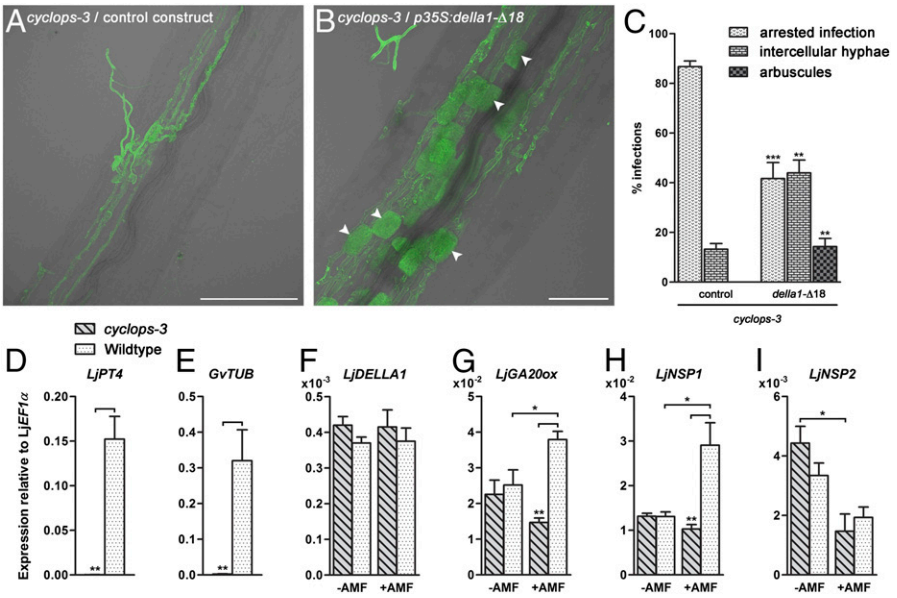

Fig. 5. AM phenotype of *cyclops* expressing *della1*-Δ18 and gene expression in *cyclops*. (*A* and *B*) Laser-scanning confocal microscope images of *G. versiforme* in *L. japonicus cyclops-3* roots expressing a control construct (*A*) or *p35S:della1*-Δ18 (*B*) at 26 d.p.i. Arrowheads mark arbuscules. Arbuscule formation is restored in *cyclops* expressing *p35S:della1*-Δ18. (Scale bars: 250 μm in *A*; 100 μm in *B*). (*C*) Percentage of infected root sections containing arrested infections, intercellular hyphae, or arbuscules. The growth of intercellular hyphae is increased significantly in roots expressing *p35S:della1*-Δ18 relative to roots expressing a control construct. Data are averages ± SEM ($n \geq 7$, where *N* denotes the number of independent root samples). (*D–I*) Gene expression in roots of *L. japonicus cyclops* and wild-type plants either mock-inoculated (–AMF) or colonized by *G. versiforme* (+AMF) at 8 wk post planting (w.p.p.), assayed by quantitative RT-PCR. Data are averages ± SEM ($n \geq 4$, where *N* denotes the number of independent root samples). $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

The SSP controls the development of both AM symbiosis and RLS, and the effects of GA on RLS have been described previously (81). GA treatment of *L. japonicus* roots altered several aspects of RLS and resulted in a partial phenocopy of *nsp2*, leading to the suggestion that GA inhibits nodulation signaling between DMI3 and NSP2 (81). By expressing *della1*-Δ18 in several SSP mutants, we were able to show an intersection between DELLA and symbiosis signaling for arbuscule formation. Constitutive expression of *della1*-Δ18 partially restores arbuscule formation in a *cyclops* mutant but not in a *dmi3* mutant, thus indicating a requirement for CCAMK. One interpretation of these data is that *della1*-Δ18–driven arbuscule development is successful only when some aspects of the cortical program have been activated by CCaMK. In *dmi3* (*ccamk*) mutants the fungus fails to enter the cortex, but in *cyclops* mutants hyphal growth in the cortex occurs, suggesting partial activation of the cortical program. In *L. japonicus*, a gain-of-function *ccamk* mutant showed cellular changes in cortex which also suggest that CCAMK influences this phase of the symbiosis (80). An alternative interpretation is that arbuscule formation driven by *della1*-Δ18 has a less stringent requirement for CYCLOPS.

Both *NSP1* and *NSP2* transcript levels are reduced significantly in *della1*/*della2* relative to wild type. This finding is consistent with nodulation studies in which GA application was shown to reduce *NSP2* transcripts and suggests that DELLA proteins regulate NSP1 and NSP2 either directly or indirectly. Despite effects on their transcripts, these two GRAS factors are not essential for the increases in arbuscule formation or hyphal development driven by *della1*-Δ18. Consequently, it seems likely that DELLA proteins regulate other, as yet unknown, transcription factors to influence arbuscule formation and hyphal development. Because *della1*-Δ18 restores arbuscules in *cyclops* mutants, we propose that at least some of these factors are regulated by *cyclops*. Alternatively, because DELLA proteins are known to influence many signaling pathways (reviewed in ref. 52), overexpression of the dominant DELLA1 could promote signaling through other pathways, for example root growth- or phosphate-signaling pathways, and could sensitize the root to enable some arbuscule formation even in the absence of complete SSP signaling. An analogous situation occurs in *Arabidopsis*, in which a dominant DELLA increases sensitivity to jasmonic acid and alters interactions with plant pathogens (46, 47, 93).

In *Arum*-type symbioses, arbuscules are the site of Pi delivery to the plant, and in *vapyrin* mutants, where arbuscules are absent, or *mtpt4* mutants, where arbuscules are not functional, intercellular hyphal growth is reduced substantially, and the hyphae show septa, which are a sign of death (20–22, 94). These data suggest that functional arbuscules are necessary for continued fungal development. Consistent with this notion, nutrient experiments indicate that carbon and Pi exchange are linked functionally, and a reciprocal rewards model has been proposed in which Pi delivery by the fungal symbiont is rewarded with increased carbon, and vice versa (95, 96). Given these data, the extensive

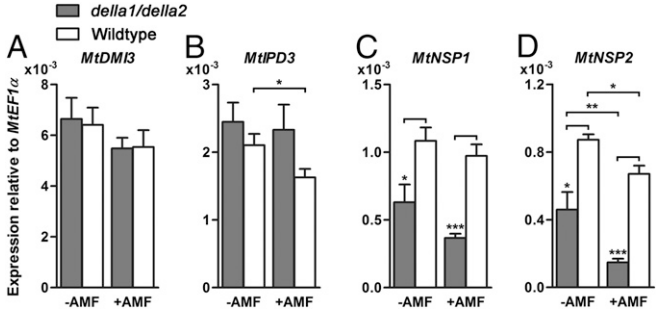

Fig. 6. Expression of SPP genes in roots of *della1*/*della2* and wild-type plants. (*A–D*) Transcript levels of SSP genes in roots either mock-inoculated (–AMF) or colonized by *G. versiforme* (+AMF) at 21 d.p.i., assayed by quantitative RT-PCR. Data are averages ± SEM ($n \geq 5$, where *N* denotes the number of independent root samples). $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

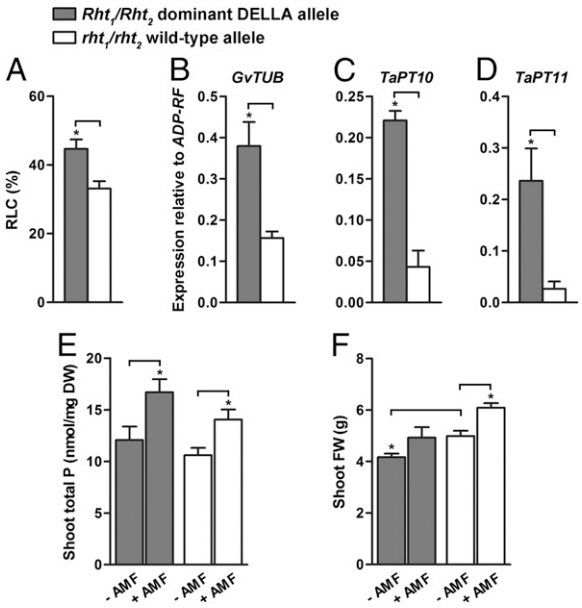

Fig. 7. AM phenotype of a Rht_1/Rht_2 dominant DELLA dwarf wheat line colonized by *G. versiforme*. (*A*) Level of colonization of the Rht_1/Rht_2 dominant DELLA dwarf and rht_1/rht_2 wild type at 31 d.p.i. The Rht_1/Rht_2 line differs significantly from the rht_1/rht_2 wild-type line. Data are averages ± SEM (n = 3, where N denotes the number of independent root samples). (*B*–*D*) Gene expression of *G. versiforme* α-*tubulin* (*GvTUB*) (*B*) and the phosphate transporters *TaPT10* (*C*) and *TaPT11* (*D*) in dwarf and wild-type roots at 31 d.p.i. relative to the *Triticum aestivum ADP ribosylation factor* (*ADP-RF*) assayed by quantitative RT-PCR. *TaPT10* and *TaPT11* transcript levels are significantly higher in the dwarf than in the wild-type roots. Data are averages ± SEM (n = 3, where N denotes the number of independent root samples). (*E*) Phosphorus content in shoots of noncolonized (–AMF) and colonized (+AMF) dwarf and wild-type plants at 13 wk post inoculation (w.p.i.). Data are averages ± SEM (n = 4, where N denotes the number of independent plants). DW, dry weight. (*F*) Shoot fresh weight (FW) of noncolonized and colonized dwarf and wild-type plants at 13 w.p.i. Data are averages ± SEM (n = 4, where N denotes the number of independent plants). $^*P \leq 0.05$.

growth of intercellular hyphae in *della1/della2* and in GA-treated roots was surprising, because the fungus proliferates in the cortex without forming arbuscules. This phenotype suggests that the symbiosis-associated link between phosphate and carbon has been uncoupled in this mutant. It is known that GA signaling can have direct effects on starch metabolism (97, 98). During AM symbiosis, starch decreases as the roots become colonized (99). It is possible that in the *della1/della2* mutant, constitutive GA signaling may promote starch degradation, enabling the fungus to proliferate in the cortex in the absence of symbiotic Pi delivery.

Studies in *Arabidopsis* indicate that DELLA proteins influence signaling through multiple pathways and coordinate aspects of growth, development, and also responses to defense and to various abiotic stresses (51, 52). In general DELLA proteins act as repressors of GA signaling and negatively regulate plant growth and development through interactions with a myriad of transcription factors. In addition, they respond to environmental signals and mediate cross-talk with other hormone-signaling pathways, enabling the coordination of growth with adaptive responses to abiotic and biotic stresses. AM symbiosis is one of the less common examples in which DELLA proteins appear to function as positive regulators; a similar observation has been made for plant interactions with biotrophic pathogens that are promoted by DELLA proteins as a result of modulation of jasmonic acid and salicylic acid signaling (47).

There is considerable evidence that the plant coordinates the development of AM symbiosis with its nutritional requirements, carbon availability, and root growth (33, 100–103). Based on our current data and DELLA proteins' established roles in growth restraint as well as cross-talk with other signaling pathways (43, 52, 53), we propose that DELLA proteins provide a mechanism to coordinate arbuscule formation and AM symbiosis with plant nutrient status and growth and that DELLA-mediated regulation of symbiosis occurs, in part, through regulation of the SSP. As outlined in Fig. 8, during Pi-limiting conditions, DELLA expression is high, and arbuscule formation is promoted through effects on unknown transcription factors that lie downstream of *cyclops* in the SSP. Once arbuscules are formed, symbiotic Pi transport leads to an increase in Pi levels which negatively regulate DELLA transcription. In addition, symbiosis-induced and/or Pi-induced expression of GA biosynthesis genes results in a rise in GA levels that leads to the proteolytic degradation of DELLA; consequently, negative feedback regulates arbuscule formation. Control of GA levels through a combination of biosynthesis and degradation provides a mechanism for regulating and fine-tuning GA levels and consequently DELLA activity in the mycorrhizal root. In addition, GA transport may further regulate cellular GA levels, although the proteins involved in this process are as yet unknown (104). The current data provide evidence that DELLA proteins control arbuscule formation, and, although the precise mechanism remains to be determined, the role of DELLA proteins as positive regulators of arbuscule formation coupled with their position at the nexus of many signaling pathways provides a mechanism for balancing symbiosis with plants' nutritional needs, growth, and development.

Materials and Methods

Plant and AM Fungal Growth Procedures. Unless otherwise stated, plants were grown in a growth chamber under a 16-h light (25 °C)/8-h dark (22 °C) regime at 40% relative humidity in sterile Turface (Profile Products) inoculated with 300 surface-sterilized *G. versiforme* or *G. intraradices* spores per plant, as described (105), and fertilized once a week with modified half-strength Hoagland's solution containing full-strength nitrogen and 20 μM potassium phosphate.

To characterize the AM phenotype in *della1/della2* plants (Fig. 1 *A*–*C*, *E*, and *F*), 2-d-old seedlings were planted in a sand layer 4 cm below the top of 20.5-cm cones filled with a sterile gravel/filter sand mixture (1:1 ratio) containing 300 surface-sterilized *G. versiforme* spores. Seedlings were fertilized every third day with the above-mentioned fertilizer solution. Transcript analyses (Figs. 1*D*, 2, and 6), spore counting (Table 1), and plant phenotype

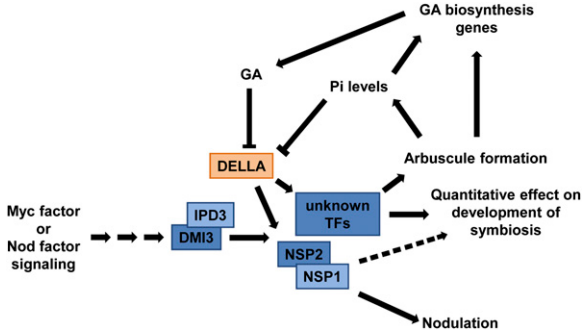

Fig. 8. A model integrating DELLAs and AM symbiosis. In Pi-deprived plants, DELLA transcript levels are high (Fig. S3) (71), and DELLA promotes arbuscule formation and hyphal growth in the cortex (Figs. 1 and 3–5) through the activation of as yet unknown transcription factors (TFs). Arbuscule development and the resulting symbiotic Pi transport leads to an increase in Pi levels in the root (94), resulting in a decrease in DELLA transcript levels (Fig. S3) (71). Genes of GA biosynthesis and GA inactivation are induced during symbiosis, indicating complex regulation of bioactive GA levels (Fig. 2*D*) (35). GA levels rise in the roots (40), leading to the degradation of DELLA protein. A decrease in DELLA (transcripts and protein) regulates arbuscule development through negative feedback. In plants with a high Pi status, low levels of DELLA proteins result in minimal arbuscule formation.

evaluation (Fig. S4) were performed on plants growing in cones filled with a sterile gravel/sand mixture (1:2 ratio) inoculated with 500 surface-sterilized *G. versiforme* spores per cone. Plants were fertilized daily with above-mentioned fertilizer solution. Plants were harvested 21 d post inoculation.

The data shown in Fig. S5 were generated from plants grown in a double-cone system to synchronize infection (106), with the following modifications. Two seedlings were used per cone, and the cones contained sterile sand. Plants were fertilized once a week with above-mentioned fertilizer. After 2 wk, the plant cone was placed over the spore cone, which was partially filled with a sterile Turface/sand mixture (1:1 ratio) with a 1-cm sand layer at the top of the mixture. Eight hundred sterile *G. versiforme* spores were spread onto this sand layer. After 12 d, the nylon mesh was removed, and the plant cone was pushed down. Cones were harvested 7, 9, and 11 d after the physical contact of the roots with *G. versiforme* spores.

To analyze gene expression in *L. japonicus* Gifu wild-type and *cyclops-3* roots, 7-d-old seedlings were planted in cones filled with sterile filter sand containing 500 surface-sterilized *G. versiforme* spores and were fertilized every third day with modified half-strength Hoagland's solution containing full-strength nitrogen and 20 μM potassium phosphate. Plants were harvested 8 wk post planting.

Three-week-old composite *L. japonicus* plants were inoculated with 300 surface-sterilized *G. versiforme* spores and were grown in 11-cm pots filled with a sterile gravel/filter sand mixture (1:1 ratio). Plants were harvested 26 d post inoculation.

For analysis of AM symbiosis in wheat (Fig. 7 *A*–*D*), 3-d-old seedlings were transplanted to 11-cm pots containing sterile gravel/filter sand mixture (1:1 ratio) and were inoculated with 400 surface-sterilized *G. versiforme* spores. To monitor mycorrhiza-associated growth response (Fig. 7 *E* and *F*), seedlings were grown in a sterile gravel/filter sand mixture (1:1 ratio) with 400 surface-sterilized *G. versiforme* spores per seedling and were fertilized weekly with modified half-strength Hoagland's solution containing full-strength nitrogen and 20 μM potassium phosphate. After 3 wk, plants were transferred to a sterile Lansing soil/sand mixture (1:5 ratio) and were fertilized twice a week with a modified 1/4× Hoagland's solution containing half-strength nitrogen and without potassium phosphate. Once a week, the fertilizer was supplemented with 10 mL 0.5 mM $Ca_3(PO_4)_2$ as described (107). The plants were harvested 10 wk post transfer.

To visualize fungal structures, roots were stained in 0.2 mg/mL WGA Alexafluor 488 (Molecular Probes) (94). Colonization levels specified as percent root length colonized were assessed by the modified gridline intersect method (108). Image J software was used to measure the infected root length. Infected root lengths could contain more than one infection unit. Arbuscule density was quantified by counting arbuscules within fungal colonization units (Fig. 1*C*) or by counting arbuscules within a defined area (Figs. S5*B*, S8*C*, and S10*C*).

Spore production was assessed by counting the numbers of spores in 1-cm^2 areas randomly across the root system. Roots were harvested 7 wk after inoculation. Five independent samples of *della1/della2* mutant and wild-type roots were analyzed.

GA Treatment. A 50-mg/mL gibberellic acid stock solution (GA_3; G-7645; Sigma) was created with ethanol and diluted with H_2O_{dd} to a working solution of 10^{-6} M GA_3. Control pots received H_2O_{dd} with the equivalent volume of ethanol (solvent solution). Starting 6 d post inoculation, 50 mL GA_3 working or solvent solution was applied daily to each 11-cm pot.

Cloning and Vector Construction. *pMtDELLA1:UidA* was created by amplifying a 1,054-bp fragment containing the region 5′ proximal to the *MtDELLA1* ATG start codon with primers that added 5′ SalI and 3′ HindIII restriction sites (Table S1). The *MtDELLA1* promoter fragment was inserted between the SalI and HindIII restriction sites of a modified pCAMBIA2301 vector that lacked the CaMV 35S-promoter (109).

The dominant *DELLA1* gene, *della1*-Δ18, was made by fusion PCR to create a gene with a 54-nt deletion, which results in a DELLA protein lacking 18 amino acids encompassing the DELLA motif, beginning at amino acid 60 (MDELLAALGYKVRSSDMA). The deletion was designed based on the *Arabidopsis* mutants *gai-1* and *rga-Δ17*, which contain a 17-aa deletion of the DELLA motif (55, 110). The resulting *della1*-Δ18 gene was inserted between the SalI and BsrGI restriction sites of a pCAMBIA2300 vector that contains *p35S:GFP* (pJL33) to create *p35S:della1*-Δ18. pJL33 was created by inserting a HindIII-EcoRI fragment containing the CaMV 35S-promoter-sGFP-AtNOS fusion from the CaMV35S-sGFP(S65T)-Nos plasmid (111) into its multiple cloning site. pJL33 was used as vector control for the experiment.

To create *pMtDELLA1:GFP-della1*-Δ18, *della1*-Δ18 was amplified with primers that added 5′ BsrGI and 3′ PmlI/NotI restriction sites. The fragment was digested with BsrGI and NotI and was ligated into the CaMV35S-sGFP(S65T)-Nos plasmid, resulting in an in-frame 3′ fusion to GFP driven by the CaMV 35S-promoter. This plasmid was digested with PstI and PmlI and was ligated into pCAMBIA2301, resulting in *p35S:GFP-della1*-Δ18. The *MtDELLA1* promoter was amplified with primers adding 5′ and 3′ SalI restriction sites and was inserted in 5′-to-3′ orientation between the two SalI restriction sites of *p35S:GFP-della1*-Δ18, replacing the CaMV 35S-promoter. *pMtPT9:della1*-Δ18 was created by amplifying the *MtPT9* promoter with primers that added 5′ BamHI and 3′ SalI restriction sites. The *MtPT9* promoter fragment (Fig. S7) was inserted between the BamHI and SalI restriction sites of *p35S:della1*-Δ18, replacing the CaMV 35S-promoter. To enable identification of transgenic roots, the *Ubiquitin* promoter: dsRED1 reporter cassette from the modified pHellsgate 8 vector was blunt end-cloned into *pMtPT9:della1*-Δ18 and *p35S:della1*-Δ18 using the BamHI restriction sites of these vectors. pJL33 was used as vector control for the experiment.

***Agrobacterium rhizogenes*-Mediated Transformation.** Composite *M. truncatula* plants were produced by *Agrobacterium rhizogenes*-mediated transformation (112, 113) with minor modifications as described in refs. 20 and 26. To generate composite *L. japonicus cyclops-3* plants, constructs were transferred via *A. rhizogenes* strain AR1193 as described (114).

Genotyping of *Tnt1* Insertion Lines and Generation of *della1/della2*. *M. truncatula* R108 *Tnt1* transposon insertion lines in DELLA1 (NF5155 and NF4215) and DELLA2 (NF4302) were obtained from The Noble Foundation (Fig. S3*E*). R1 plants were grown and genotyped by PCR using a transposon-specific primer (Tnt1-F) and *DELLA1* or *DELLA2* gene-specific primers (Table S1). Wild-type plants at the *DELLA1* or *DELLA2* loci were identified using the corresponding gene-specific primers. *della1* alleles were backcrossed to R108. Backcrossed *della1* alleles from NF5155 and NF4215 were crossed with *della2* to create two *della1/della2* double mutants. The *della1/della2* double mutants show a similar plant and AM phenotype. All comparative experiments were performed with the *della1/della2* mutant obtained from the NF5155 *della1* allele and included wild-type segregants from the respective *della1* and *della2* populations as controls.

RNA Isolation, cDNA Synthesis, and Semiquantitative and Quantitative RT-PCR. Unless otherwise stated, RNA isolation, cDNA synthesis, and PCR were carried out as described previously (35). Quantitative real-time RT-PCR was performed as described (34). Total RNA from *L. japonicus* roots was extracted as described in ref. 30.

Determination of Phosphorus Content, Histochemical Staining for GUS, and Laser-Scanning Confocal Microscopy. Phosphorus content was determined by a phosphomolybdate colorimetric assay (115).

Histochemical staining for GUS activity was performed as previously described (20).

Laser-scanning confocal microscopy was carried out as described previously (73).

Statistical Analyses. Unless otherwise stated, the Kruskal–Wallis rank sum test was applied.

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