Gibberellins Interfere with Symbiosis Signaling and Gene Expression and Alter Colonization by Arbuscular Mycorrhizal Fungi in *Lotus japonicus*

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Arbuscular mycorrhiza is a mutualistic plant-fungus interaction that confers great advantages for plant growth. Arbuscular mycorrhizal (AM) fungi enter the host root and form symbiotic structures that facilitate nutrient supplies between the symbionts. The gibberellins (GAs) are phytohormones known to inhibit AM fungal infection. However, our transcriptome analysis and phytohormone quantification revealed GA accumulation in the roots of *Lotus japonicus* infected with AM fungi, suggesting that de novo GA synthesis plays a role in arbuscular mycorrhiza development. We found pleiotropic effects of GAs on the AM fungal infection. In particular, the morphology of AM fungal colonization was drastically altered by the status of GA signaling in the host root. Exogenous GA treatment inhibited AM hyphal entry into the host root and suppressed the expression of inferred GA signaling, whereas our study demonstrates that GA signaling also positively interacts with symbiotic responses and promotes AM colonization of the host root.

Arbuscular mycorrhiza develops in the host roots of leguminous plants infected with AM fungi. AM fungi enter the host root and form a symbiotic organ called a nodule, where the bacterial symbionts (rhizobia) fix atmospheric nitrogen into ammonia and thus provide the host plant with nitrogen (Oldroyd et al., 2011). RNS evolved by the recruitment of several AM genes and systems of the host plant (Oldroyd and Downie, 2008), and a molecular mechanism shared between arbuscular mycorrhiza and RNS, termed the common symbiosis pathway, plays important roles in the development of both types of symbiosis (Parniske, 2008).

Arbuscular mycorrhiza development is controlled by both AM fungi and the host plant. Symbiosis signaling molecules derived from both the host plant and AM fungi contribute to recognition between the symbionts and activate symbiosis signaling pathways (Akiyama et al., 2005; Besserer et al., 2008; Maillet et al., 2011; Czaia et al., 2012). Following the initial signal exchanges, the AM hyphae enter the host root and colonize the cortical cell layers. The AM fungus *Rhizopagus irregularis* shows Arum-type hyphal elongation in the roots of the leguminous model plant *Lotus japonicus* (Demchenko et al., 2004). In Arum-type colonization, the AM fungal hyphae elongate between the host root cells and often branch in the host cortex. A branched hypha grows between or penetrates into the host cells and continues to elongate, which...
increases hyphal density in the host root. The penetrated hypha often continues to branch inside the host cell and differentiates into an arbuscule. Before hyphal penetration into the host cells, characteristic intracellular remodeling (such as enlargement of the nucleus, development of the endoplasmic reticulum, and enlargement of the cytosolic space) occurs in the epidermal and cortical cells near the AM hyphae (Genre et al., 2005). The prepenetration apparatus (PPA) is a preinfection structure for fungal penetration; PPA formation is triggered by symbiosis signals transmitted through the common symbiosis pathway (Genre et al., 2005).

Calcium- and calmodulin-dependent protein kinase (CCaMK) is a key component of the common symbiosis pathway (Lévy et al., 2004; Gleason et al., 2006; Tirichine et al., 2006). CCaMK is composed of a kinase domain and regulatory domains that include a calmodulin-binding domain and EF-hand motifs (Patil et al., 1995; Takezawa et al., 1996). The kinase activity is controlled by the regulatory domains; therefore, removal of these domains releases inhibition and stimulates the kinase activity, thus conferring gain-of-function properties (Takeda et al., 2012). The gain-of-function calcium- and calmodulin-dependent protein kinase (GOF-CCaMK) constitutively activates a part of the downstream symbiosis signaling pathway that induces arbuscular mycorrhiza- and RNS-induced gene expression and the formation of PPA-like structures without AM fungal infection. Thus, the host plant regulates AM colonization, including hyphal branching and arbuscule formation, by the common symbiosis pathway and PPA formation.

Symbiotic microbe infection is also regulated by plant hormones. GAs, strigolactones, cytokinins, ethylene, and brassinosteroids affect developmental processes in arbuscular mycorrhiza and RNS (El Ghachtouli et al., 1996; Penmetsa and Cook, 1997; Ferguson et al., 2005, 2011; Floss et al., 2013; Foo et al., 2013). GAs inhibit the formation of the infection thread and nodules in RNS (Maekawa et al., 2009), and recent genetic studies revealed that abnormal elevation of GA signaling decreases colonization of the host root by AM fungi (Floss et al., 2013; Foo et al., 2013). Thus, GA signaling negatively affects AM fungal infection and development. The diterpenoid plant hormones are synthesized by consecutive reactions catalyzed by oxidases, such as ent-kaurene oxidase, GA 20-oxidase, and GA 3-oxidase, which catalyze the biosynthesis of active GA forms such as GA₁ and GA₃ (for review, see Hedden and Thomas, 2012). GA biosynthesis is regulated by negative and positive feedback mechanisms, which maintain GA signaling homeostasis in the plant (Hirano et al., 2008; Rieu et al., 2008; Hedden and Thomas, 2012). GAs induce the degradation of DELLA transcription regulators, which are negative regulators of GA signaling, and elicit downstream GA-specific responses (Pysh et al., 1999). DELLA proteins, containing a highly conserved DELLA domain and a GAI, RGA, and SCR (GRAS) domain, have been identified in several plant species and include GIBBERELLIN-INSENSITIVE (GAI) and REPRESSOR OF ga1-3 (RGA) from Arabidopsis (Arabidopsis thaliana) and SLENDER RICE1 (SLR1) from rice (Oryza sativa; Peng et al., 1997; Silverstone et al., 1998; Ikeda et al., 2001). The DELLA domain interacts with the GA receptor GIBBERELLIN-INSENSITIVE DWARF1 in a GA-dependent manner, which triggers degradation of the DELLA protein by the 26S proteasome pathway (Willige et al., 2007; Murase et al., 2008). The gai mutant of Arabidopsis has a deletion in the DELLA domain, which causes the loss of GA-induced degradation of the GAI protein and constitutive suppression of GA signaling (Peng et al., 1997). Thus, DELLA proteins are key regulators of GA signaling in the plant.

It has been suggested that GA signaling contributes to arbuscular mycorrhiza and RNS despite its negative effects on symbioses (El Ghachtouli et al., 1996; Ortu et al., 2012; Song et al., 2012). Here, we report the up-regulation of GA biosynthesis and the accumulation of GAs in the roots of L. japonicus during arbuscular mycorrhiza development. An elevated GA level inhibited the expression of some arbuscular mycorrhiza-induced genes and AM fungal infection, in line with previous reports (El Ghachtouli et al., 1996; Floss et al., 2013; Foo et al., 2013). Moreover, our studies revealed novel aspects of GA function in arbuscular mycorrhiza: we found that GA signaling promotes AM fungal colonization associated with symbiosis signaling and arbuscular mycorrhiza-induced gene expression.

RESULTS

GA Biosynthesis Genes Are Up-Regulated during Arbuscular Mycorrhiza

We analyzed the transcriptome of L. japonicus roots inoculated with R. irregularis by RNA sequencing (RNA-Seq) to examine the alternation of GA biosynthesis gene expression during arbuscular mycorrhiza. The up- and down-regulated genes during arbuscular mycorrhiza identified in the transcriptome analysis (false discovery rate < 0.001) are listed in Supplemental Table S1. The AM marker genes arbuscular mycorrhiza-induced subtilisin-like serine protease1 (SbtM1) and Phosphate transporter4 (PT4) and other homologs of known arbuscular mycorrhiza-induced genes (RAM1 [chr1. CM1852.30.r2; Gobbato et al., 2012], two RAM2 genes, Glycerol-3-phosphate acyltransferase1 [GPAT1; CM0905.50.r2] and GPAT2 [CM0905.160.r2; Wang et al., 2012], and Vypartin1 [Vyp1]; Pumplin et al., 2010]), chitinase, lipase, and germin-like protein genes were up-regulated in the host root (Table I; Supplemental Table S1), indicating that our transcriptome analysis detected arbuscular mycorrhiza-induced genes. A number of GA biosynthesis gene homologs were up- or down-regulated during arbuscular mycorrhiza (Table I), including ent-kaurene oxidase, GA 20-oxidase (GA2ox), and GA 3-oxidase (GA3ox) homologs. Homologs of GA 2-oxidases (GA2ox), which metabolize or degrade the active GA forms, also
were induced in the host root during arbuscular mycorrhiza. The up-regulation of \textit{GA3ox1}, \textit{GA20ox1}, \textit{GA20ox2}, \textit{GA2ox1}, and \textit{GA2ox2} was confirmed by real-time quantitative reverse transcription (qRT)-PCR. The arbuscular mycorrhiza-induced genes \textit{SbtM1}, \textit{RAM1}, \textit{GPAT1}, and \textit{Vpy1} were highly induced in the infected roots compared with uninoculated roots at 3 weeks after inoculation (wai), whereas the RNS-specific gene \textit{Nodule inception (NIN)} was not induced (Fig. 1A). The induction of the GA biosynthesis and metabolism gene homologs was statistically significant ($>3.9$ [\textit{GA3ox1}], $>3.5$ [\textit{GA20ox1}], $>6.1$ [\textit{GA20ox2}], $>2.6$ [\textit{GA2ox1}], and $>5.5$ [\textit{GA2ox2}]; $n = 3$–5; Mann-Whitney \textit{U} test, $P < 0.05$; Fig. 1B), although the induction levels were much lower than those of the AM marker genes (Fig. 1A). This difference in the expression levels is in line with the data obtained by RNA-Seq analysis (Table I).

To confirm that the GA biosynthesis and metabolism genes are induced by symbiosis signals, we analyzed their induction in transgenic hairy roots carrying GOF-CCaMK (CCA3414-3'-NLS) under the control of the \textit{CaMV 35S} promoter.
of the cauliflower mosaic virus (CaMV) 35S promoter (Takeda et al., 2012). Gene expression in roots carrying CCaMK314TD-NLS was analyzed by real-time qRT-PCR and normalized to that in transgenic roots carrying the vector control. In the roots carrying CCaMK314TD-NLS and a cotransformation marker, DsRED, both arbuscular mycorrhiza- and RNS-induced genes (SbtM1, RAM1, GPAT1, Vpy1, and NIN) were up-regulated in the absence of infection (Fig. 1C). The GA biosynthesis and metabolism genes (except GA2ox2) also were significantly induced by CCaMK314TD-NLS in comparison with the vector control (36.2 [GA3ox1], 33.8 [GA20ox1], 7 [GA20ox2], 26.4 [GA2ox1], and 0.4 [GA2ox2], n = 4; Mann-Whitney U test, P < 0.05; Fig. 1D). This indicates that these genes are induced by the symbiosis signaling pathway downstream of CCaMK, although we cannot exclude that other stimuli or secondary effects also may induce these genes.

GA Accumulation in the Host Root Revealed by Quantification of Plant Hormones

Transcriptome analysis suggested that GA biosynthesis and metabolism are activated during AM fungal infection, which may result in an increase in GA concentrations in the host root. To determine the endogenous concentrations of GAs in arbuscular mycorrhiza-infected roots, we measured the levels of GAs and other phytohormones by ultra-performance liquid chromatography coupled with tandem quadrupole mass spectrometry with electrospray interface (UPLC-ESI-qMS/MS; Kojima et al., 2009). Although some hormones measurable by UPLC-ESI-qMS/MS were below the detection limit, several derivatives of GAs, cytokinin (isopentenyl adenine), auxin (indole-3-acetic acid), abscisic acid, jasmonic acid, and salicylic acid, were detected (Table II). The levels of all hormones except for GAs were not significantly different in uninfected and AM fungi-colonized roots (Table II). GA19, GA20, and GA53 (inactive GA intermediates), GA1 (an active GA synthesized from the inactive intermediates by GA 20-oxidase and GA 3-oxidase), and GA8 (an inactive product of GA1 degradation by GA 2-oxidase) were detected in this analysis. GA1, GA8, GA19, and GA53 accumulated in the infected roots at significantly higher levels than in uninfected roots (Table II).

Histochemical Expression Analysis of GA Biosynthesis and Metabolic Genes

To survey the regions of the root where GA concentrations are elevated during arbuscular mycorrhiza development, we analyzed local and tissue-specific expression of the GA biosynthesis and metabolism genes. The GUS gene was fused with promoter fragments of the genes for GA 20-oxidases up-regulated during arbuscular mycorrhiza (GA20ox1 and GA20ox2) and one of the GA 2-oxidase genes (GA2ox2), and the GUS activities were analyzed during AM fungal infection.
Weak GUS staining (background expression) was observed around the vascular bundles in uninfected transgenic roots carrying the promoter-GUS fusions (Supplemental Fig. S1). When *R. irregularis* colonized the host root, GUS activity of the GA20ox2 promoter fusion was observed in the root cells around inner hyphae (Fig. 2A). Cortical cells containing arbuscules showed particularly strong GUS staining in comparison with the neighboring cells (Fig. 2B). This staining pattern induced by fungal infection was almost identical to those in transgenic roots carrying the GA20ox1 or GA2ox1 promoter fusion (Fig. 2, C and D), although the staining time required to detect GUS, which reflects promoter activity, was different for different constructs (2 h for GA20ox1, 4 h for GA20ox2, and 6 h to overnight for GA2ox1). These results indicate that the genes involved in GA biosynthesis (GA20ox1 and GA20ox2) and metabolism (GA2ox1) are up-regulated in the same tissues and cells and that the elevation of GA concentration occurs in the cells that are in direct contact with or close to the fungal hyphae during arbuscular mycorrhiza development.

Effects of Exogenous GA on AM Fungal Colonization

To analyze GA function in AM fungal infection, we used a pharmacological approach and examined the effects of exogenous active GA (GA$_3$) or a GA biosynthesis inhibitor (uniconazole-P), which are known to be biologically active in RNS in *L. japonicus* (Maekawa et al., 2009).

GA$_3$ reduced hyphal colonization and arbuscule formation in a dose-dependent manner (Fig. 3, A and B), in line with previous reports (El Ghachtouli et al., 1996; Yu et al., 2014). The highest concentration of GA$_3$ reduced hyphal entry into the host root (Fig. 3C), indicating that this is the likely major cause of the reduction in root length colonization by the AM fungi. In contrast, GA$_3$ increased hyphal branching in the cortical cell layer compared with untreated roots (Fig. 3, D–G; Table III). In the GA-treated roots, the ratio of infected to uninfected regions was much lower than in the control roots (Fig. 3A) and arbuscule formation was hardly detectable (Fig. 3, B and G); however, in the infected regions, the cortex was filled with finely branched hyphae (Fig. 3, F and G) and many of them showed intracellular branching (Supplemental Fig. S2). To quantify the effect of GA$_3$ on hyphal density (hyphal number along the short axis of the root) in the host roots, we counted branched and elongating hyphae in the tip region of the inner hyphae (Supplemental Fig. S3). The number of branched hyphae increased significantly in the roots treated with GA$_3$ (Table III; Supplemental Fig. S3, B and C), which indicates that GA treatment increased hyphal branching and density in the cortical cell layers.

Suppression of GA Biosynthesis or Signaling Reduces Hyphal Branching

The GA levels in uniconazole-P-treated roots (infected and uninfected) were reduced to below the detection limit. Hyphal entry and root length colonization, measured as the hyphal presence per unit of root length, were not affected by uniconazole-P (Fig. 3, A and C). However, a high concentration of uniconazole-P (10$^{-6}$ M; Fig. 3, H and I) induced a severe reduction in hyphal branching in the host roots, and often only a single hypha elongated in the host root. The reduced hyphal branching caused the low hyphal density and arbuscule formation in the cortical cell layers (Table III; Fig. 3, B, H, and I). The reduction of branching by uniconazole-P could be rescued...
by GA3 (Supplemental Fig. S3E), which demonstrates that alterations of GA concentration are sufficient to change the density and form of AM hyphae in the host root. On the other hand, the reduction in arbuscule formation in the roots treated with uniconazole-P was not complemented by GA3 treatment.

Uniconazole-P was reported to have antifungal activity against several species (Fletcher et al., 1986). However, \textit{R. irregularis} spore germination was not affected by uniconazole-P or GA3 treatment (Supplemental Fig. S4). Uniconazole-P also did not affect hyphal entry into the host root and hyphal elongation in the host cortical cell layer (Fig. 3, A and C). Hyphal branching reduced by uniconazole-P was recovered by GA3 treatment (Table III; Supplemental Fig. S3E). Thus, uniconazole-P appears not to affect AM fungal growth in the host root. These results indicate that uniconazole-P has no fungicide activity under our experimental conditions.

**Figure 3.** Effect of alterations in GA concentration on AM colonization. A and B, AM colonization levels of hyphae (A) and arbuscules (B) in the roots treated with GA3 or uniconazole-P were measured at 3 wai with \textit{R. irregularis}. Error bars indicate SD (n = 5–7). Asterisks indicate statistically significant differences between treated and control roots (Student’s t test, P < 0.05). The data sets shown are representative of six independent experiments. C, The numbers of hyphal entry points per root length in the host roots treated with GA3 or uniconazole-P were calculated. The entry points (where AM hyphae attached and entered the root) were counted at 1 wai with \textit{R. irregularis}. Error bars indicate SD (n = 19–37). D to I, AM fungi in the host roots (3 wai) treated with mock solution (D and E), 10^{-6} M GA3 (F and G), or 10^{-6} M uniconazole-P (H and I) were stained with WGA-Alexa Fluor 594. E, G, and I are magnifications of the areas within the white rectangles in D, F, and H, respectively. Arrowheads in E indicate arbuscules in the host cortical cells. Control, Mock-treated root; GA3, GA3-treated root; Uni, uniconazole-P-treated root. Bars = 1 mm (D, F, and H) and 200 \mu m (E, G, and I).

**Table III.** Numbers of branched hyphae elongating along the longitudinal axis in the tip region

<table>
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<tr>
<th>Parameter</th>
<th>Control</th>
<th>GA3 (10^{-6} M)</th>
<th>Uni (10^{-6} M)</th>
<th>Uni + GA (10^{-6} M each)</th>
<th>Vector Control</th>
<th>GA3, delta-GA1</th>
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<tr>
<td>No. of hyphae</td>
<td>5.1 (2.4, 50)</td>
<td>7.4* (3.5, 34)</td>
<td>3.7* (2.4, 20)</td>
<td>5.9 (2.7, 40)</td>
<td>5.3 (2.6, 80)</td>
<td>3.9* (2.3, 89)</td>
</tr>
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Uniconazole-P may inhibit other biosynthesis processes in the host root (Iwasaki and Shibaoka, 1991). Therefore, we investigated whether its effects could be mimicked in the AM fungi-infected transgenic roots with GA signaling constitutively suppressed by a dominant-negative DELLA transcription regulator. We identified an *L. japonicus* GAI homolog (LjGAI) that contains the conserved DELLA and GRAS domains (61% and 56% overall amino acid sequence identity to AtGAI and SLR1, respectively; Floss et al., 2013). To confer the dominant-negative property on LjGAI, we deleted five amino acid residues in its DELLA domain (delta-GAI; Supplemental Fig. S5A). Overexpression of delta-GAI under the control of the CaMV 35S promoter (35S_pro:delta-GAI) in the hairy roots of *L. japonicus* caused their swelling and severe growth inhibition (Supplemental Fig. S5, B and C), which are typical responses to the suppression of GA signaling (Ubeda-Tomás et al., 2008). This phenotype was similar to that observed in uniconazole-P-treated roots (Supplemental Fig. S5B). The morphology of more than 80% of the transgenic roots was affected (Supplemental Fig. S5B), whereas the remaining roots were either slightly thicker and shorter or indistinguishable from nontransgenic roots. The swollen roots showed a severe reduction in AM fungal infection (Supplemental Fig. S6, A and B). However, it is unclear whether the reduction in AM colonization was caused by the suppression of GA signaling or by shorter roots. Therefore, we created a construct for delta-GAI production driven by the native *LjGAI* promoter (GAI_pro:delta-GAI) that was expected to have weaker promoter activity than the CaMV 35S promoter and used it for the analysis of AM fungal colonization in the roots. GAI_pro:delta-GAI still often induced a swollen root phenotype (20% of the transgenic roots; Supplemental Fig. S5, B and D), although these morphological changes were weaker than those induced by 35S_pro:delta-GAI. Unlike in transgenic roots carrying 35S_pro:delta-GAI, root length colonization was not changed significantly in roots carrying GAI_pro:delta-GAI (Supplemental Fig. S6, A and B). On the other hand, GAI_pro:delta-GAI also reduced hyphal branching in the tip region of the intraradical hyphae (Table III; Supplemental Fig. S6, C and D). These effects of delta-GAI on root morphology and hyphal branching were similar to those of uniconazole-P (Table III; Supplemental Fig. S5B), indicating that the suppression of GA biosynthesis or signaling influences AM fungal branching in the host root. These results suggest that GA is not just a negative regulator for AM fungal infection but is required for hyphal colonization in the host root.

**GA Signaling Affects Arbuscular Mycorrhiza-Induced Gene Expression**

We also analyzed the effects of GA on the expression of the arbuscular mycorrhiza-induced genes SbtM1, RAM1, GPAT, and *Vpy1*, because knockdowns or knockouts of these genes affect AM colonization in *L. japonicus* and *Medicago truncatula* (Takeda et al., 2009; Pumplin et al., 2010; Gobbato et al., 2012; Wang et al., 2012). Either decreased or increased GA levels reduce AM fungal colonization in the roots (Fig. 3), which would result in decreased induction levels of the arbuscular mycorrhiza-induced genes under either condition. Therefore, we used GOF-CCaMK hairy roots, which have constitutive arbuscular mycorrhiza-induced gene expression without AM fungal infection (Takeda et al., 2012; Fig. 1, C and D), to exclude the effects of differences in hyphal colonization levels. In roots carrying CCaMK314TD-NLS treated with uniconazole-P or additionally carrying 35S_pro:delta-GAI, SbtM1 expression was significantly inhibited in comparison with the untreated transgenic roots. Vpy1 expression was not significantly affected by GA3 or uniconazole-P but was reduced in roots carrying 35S_pro:delta-GAI; the overall pattern of responses was similar to that of *SbtM1* (Fig. 4A). GA3 treatment of untransformed and uninoculated roots did not induce the expression of these genes (Supplemental Fig. S7), indicating that GA signaling alone is unable to trigger symbiosis signaling and gene expression.

The effect of GA on *SbtM1* expression was confirmed in transgenic plants carrying the *SbtM1* promoter-GUS fusion (*SbtM1_pro:GUS*). CCaMK314TD-NLS was introduced into *SbtM1_pro:GUS* plants by hairy root transformation, and the roots were treated with mock solution, GA3, uniconazole-P, or both GA3 and uniconazole-P. Higher *SbtM1* promoter activity was observed in some cortical cells than in the neighboring cells (Fig. 4C). This is a typical pattern of *SbtM1_pro:GUS* induced by GOF-CCaMK, and strongly stained cells often contain a PPA-like structure (Takeda et al., 2012). Whereas GA3 did not change the ratio of GUS-stained roots, uniconazole-P significantly reduced both GUS intensity and the staining pattern (Fig. 4, B and C). These responses were consistent with the results of real-time qRT-PCR analysis (Fig. 4A). The reduction of GUS activity and the formation of the staining pattern by uniconazole-P was reversed by GA3 (Fig. 4, B and C), similar to the GA3 suppression of the inhibitory effect of uniconazole-P on hyphal branching (Table III; Supplemental Fig. S3). This recovery proved that insufficient amounts of GAs reduced *SbtM1* expression.

In contrast, the induction of *RAM1* and *GPAT1* was severely inhibited by GA3 (Fig. 4A). The levels of *RAM1* and *GPAT1* suppression by GA3 were comparable to the levels of induction of these genes by GOF-CCaMK (Fig. 1C); thus, the induction of *RAM1* and *GPAT1* was almost completely suppressed by GA3. Delta-GAI had the same effect on *RAM1* and *GPAT1* expression as uniconazole-P treatment (Fig. 4A), indicating that the suppression of GA signaling enhances or maintains *RAM1* and *GPAT1* expression.

**DISCUSSION**

**AM Fungal Infection Induces the Biosynthesis and Accumulation of GAs**

We detected the up-regulation of GA biosynthesis genes and an increase in GA amounts in AM fungus-colonized roots of *L. japonicus* (Tables I and II; Fig. 1, B
and D). The up-regulation of GA biosynthesis and metabolism genes during arbuscular mycorrhiza also was reported in AM plants (Gomez et al., 2009; Guether et al., 2009; Ortu et al., 2012). Histochemical analyses of both arbuscular mycorrhiza-induced GA 20-oxidases (GA20ox1 and GA20ox2), suggested to be key enzymes controlling GA amounts (Middleton et al., 2012), by using GUS fusions revealed that the up-regulation of GA biosynthesis occurs around AM hyphae in the host root during AM colonization (Fig. 2, A–C). Although the GA concentration in AM fungi-colonized roots was only 1.3 to 2.5 times that in the uninoculated roots when averaged over the whole root (Table II), the site-specific expression of the GA biosynthesis genes indicates higher local accumulation of GAs in the cells around AM hyphae. The elevated GA levels are thought to enhance GA signaling in the host root cells around AM fungi.

GA Signaling Is Required for Symbiosis Gene Expression Followed by Hyphal Colonization

Excessive GA amounts or signaling inhibit fungal colonization of the host roots; therefore, GA has been known to function as a negative regulator of arbuscular mycorrhiza (El Ghachtouli et al., 1996; Floss et al., 2013; Foo et al., 2013). Here, we found that low-GA conditions also have a negative effect on AM colonization in the host roots (Fig. 3; Table III; Supplemental Figs. S3D and S6D). Such a dual effect depending on the hormone levels or developmental stages also was reported for another hormone, abscisic acid (Charpentier et al., 2014), and for other GA-related responses (Yamaguchi et al., 2014). These results suggest that GA signaling is required for AM hyphal branching and colonization of the host root. A previous study demonstrated that the reduction in the SbtM1 induction levels to 10% to 30% by RNA interference reduced AM colonization and arbuscule formation in the host roots (Takeda et al., 2009). In our experiments here, the SbtM1 expression induced by GOF-CCaMK was reduced by uniconazole-P or over-expression of delta-GAI to below 10% of the induction level (Fig. 4A). This level of suppression is sufficient to cause the same effect as in the SbtM1 RNA interference plants. Therefore, GA signaling may control hyphal density by influencing the expression of some arbuscular mycorrhiza-induced genes (including SbtM1) in the host root.

The AM fungus R. irregularis shows Arum-type hyphal elongation in L. japonicus (Demchenko et al., 2004). Branched hyphae mainly elongate in intercellular spaces between the cells or sometimes penetrate into the cells; this is accompanied by PPA formation followed by arbuscule formation or branching (Genre et al., 2005). The decrease in the formation of the PPA-like structure induced by GOF-CCaMK at low GA (Fig. 4C) can be expected to directly lead to the reduction in hyphal branching or arbuscule formation (Table III; Fig. 3, H and I). On the other hand, hyphal branching and density increased in the roots treated with GA3 (Table III), although the PPA-like structure formation was not increased significantly by GA3 treatment (Fig. 4C). At elevated GA, a large number of intracellular branches were observed in the cortex.
instead of arbuscule formation (Supplemental Fig. S2, B and C). Therefore, the increased hyphal branching might be caused by a lack of arbuscule formation, although it has not been demonstrated that arbuscule formation and hyphal branching share common initiation mechanisms.

GA Signaling Inhibits AM Hyphal Entry, Arbuscule Formation, and the Induction of Arbuscular Mycorrhiza-Induced Genes

GA signaling positively regulated SbtM1 and Vpy1 expression, yet the GA3 treatment reduced hyphal entry into the host root and arbuscule formation (Fig. 3, B and C) and strongly suppressed the induction of the arbuscular mycorrhiza-induced genes RAM1 and GPAT1 (Fig. 4A). RAM1 and RAM2 are symbiosis proteins essential for fungal infection and are highly induced during arbuscular mycorrhiza in M. truncatula (Gobbato et al., 2012; Wang et al., 2012). The ram1 and ram2 mutants of M. truncatula show reductions in the number of infection structures of AM fungi called hyphopodia on the root surface, which greatly reduces hyphal entry and colonization of the host roots. Thus, the negative effect of GA signaling on hyphal entry is most likely caused by its interference with the expression of arbuscular mycorrhiza-induced genes that control AM fungal entry at the surface of the host roots, such as RAM1 and RAM2.

Either decreased or increased GA levels reduce arbuscules in the host root (Fig. 3, B, G, and I). Thus, a more stable level of GA would be required for proper arbuscule formation than for the recovery of hyphal entry into the cell and branching. Histochemical analysis indicated that arbuscule-containing cells show the largest changes in GA biosynthesis and metabolism genes (Fig. 2). GA 2-oxidases, which degrade active GAs, were up-regulated during arbuscular mycorrhiza development (Table I; Fig. 1B) and were particularly strongly induced in the arbuscule-containing cells (Fig. 2D). The reduction in GA concentrations by arbuscular mycorrhiza-induced GA 2-oxidases would contribute to maintaining suitable GA conditions for arbuscule formation.

Functional Model of the Interference of GA Signaling with AM Signaling and Hyphal Colonization

By suppressing or stimulating GA signaling, we revealed its positive and negative effects on arbuscular mycorrhiza-induced gene expression and AM fungal colonization of the host root. We used very strong suppression or activation of GA signaling, whereas the inhibitory and stimulatory effects would be more moderate during arbuscular mycorrhiza development. Nevertheless, our analyses suggest a functional model of the role of GA signaling in symbiosis responses in the host root during arbuscular mycorrhiza development (Fig. 5). The symbiosis signal activated by CCaMK induces symbiosis responses such as the expression of SbtM1, Vpy1, RAM1, RAM2, and the GA biosynthesis and metabolism genes (Fig. 5, I). Infection by AM fungi up-regulates the GA biosynthesis metabolism pathway and activates GA signaling in the host root cells around the AM hyphae (Fig. 5, II). Stimulated GA signaling interferes with the symbiosis signaling pathway and promotes or maintains the expression of some arbuscular mycorrhiza-induced genes that are required for hyphal colonization, which facilitates hyphal branching in the host cortex (Fig. 5, III). Simultaneously, GA signaling inhibits the expression of some other arbuscular mycorrhiza-induced genes, such as RAM1 and RAM2, and thus...
inhibits AM fungal entry at the surface of the host root (Fig. 5, IV). Arbuscule formation is affected by SbtM1, Vpy1, and RAM2 expression, meaning that GAs have both positive and negative effects on this process (Fig. 5, V). The negative effect of GA signaling on arbucular mycorrhiza development seems to contradict the accumulation of GAs during arbucular mycorrhiza. However, this effect would be limited to the regions around the AM fungi and would not affect hyphal entry in other regions of the host root. Overall, this would promote effective infection of the whole root by preventing duplicated infection at the same entry points. At later stages, high concentrations of accumulated GAs would inhibit new fungal infection, which might prevent excessive infection and serve as a local regulation system for AM colonization.

GA signaling directly or indirectly affects the symbiosis signaling pathway and downstream symbiosis gene expression and has the opposite effects on the expression of SbtM1 and Vpy1 versus RAM1 and RAM2. This indicates that these two pairs of genes are regulated by different branches of the symbiotic signaling pathway downstream of CCaMK and that GA signaling interferes with both branches. GA signaling or DELLA proteins are known to cross talk with various plant hormone signaling pathways (Achard et al., 2003; Fu and Harberd, 2003; Navarro et al., 2008; Hou et al., 2010; Gallego-Bartolomé et al., 2012). A recent study revealed that SLR1, the rice DELLA protein, interacts with the strigolactone receptor DWF414 (Nakamura et al., 2013). The interaction indicates that GA signaling may interfere with the strigolactone signaling pathway that influences AM fungal colonization (Yoshida et al., 2012). Thus, the GA effect on symbiosis observed here might be indirect and caused by GA affecting other hormonal signaling pathways. SLR1 also interacts with a RAM1 homolog through the GRAS family transcriptional regulator DELLA-INTERACTING PROTEIN1 (Yu et al., 2014). Such a protein-protein interaction may provide a direct link between GA and the symbiosis signaling pathway. These signaling pathways potentially influence the symbiosis signaling pathway and alter arbucular mycorrhiza development. Spatial and temporal changes in GA signaling had different effects on AM colonization (Fig. 3; Floss et al., 2013; Foo et al., 2013), and the pleiotropic effects of GA on arbucular mycorrhiza would be caused by differential interferences that depend on the levels of GA signaling.

**MATERIALS AND METHODS**

**Plant and Fungal Materials and Growth Conditions**

Seeds of *Lotus japonicus* ‘Gifu B-129’ or ‘Miyakojima MC20’ were scarified, sterilized with sodium hypochlorite (effective chloride, 0.1%), and soaked in sterilized water overnight. The seeds were germinated on 0.8% (w/v) agar plates and grown in a growth chamber (24°C, 16 h of light/8 h of dark).

For the analysis of spontaneous symbiosis gene induction by GOF-CCaMK, plants carrying GOF-CCaMK (Takeda et al., 2012) introduced by hairy root transformation were transferred to and grown in a sterilized inoculation pot in a growth chamber (24°C, 16 h of light/8 h of dark) for 2 weeks. To prepare the inoculation pot, one plastic container (65 × 65 × 100 mm; Iwaki) was placed on the top of another container and a hole (1 cm in diameter) was made in the bottom of the top container. A Kimwipe (Crecia) was placed through the hole so that it reached the bottom of the top container. Wet expanded vermiculite (300 mL; Hakugen) was put into the top container, and Broughton and Dilworth medium containing 0.1 mM KNO₃ (100 mL) was supplied to the top container. The medium that flowed through the hole into the bottom container was later taken up to the soil in the top container along the Kimwipe.

The AM fungus *Rhizophagus irregularis* (DAOM197198; PremierTech) was propagated in a chive (*Allium schoenoprasum*) nurse pot system according to Demchenko et al. (2004), except that one-tenth-strength Hoagland solution (pH 5.5) and a mixture (300 mL) of river sand and expanded vermiculite (1:1, v/v) in the inoculation pot were used. After removal of the chive shoots, the pots were used for AM fungal inoculation. Inoculated plants were supplied with 100 mL of one-tenth-strength Hoagland solution containing 0.1 mM KNO₃ and grown in a growth chamber (24°C, 16 h of light/8 h of dark). GA₃ and uniconazole-P (both from Wako) were diluted to 10⁻⁶, 10⁻⁵, or 10⁻⁴ M with one-tenth-strength Hoagland solution or Broughton and Dilworth medium (each supplemented with 0.1 mM KNO₃). The hairy root transgenic plants or seedlings (16–32 plants per pot) were transplanted into the chive nurse pots or sterilized inoculation pots described above. Medium containing diluted GA₃, uniconazole-P, or both (100 mL per pot) was poured directly over the soil and the plants. The diluents were used as mock controls. The plants were removed grown in a growth chamber (24°C, 16 h of light/8 h of dark).

Germination of *R. irregularis* spores was tested after 1 week of incubation (28°C) on Bécard’s M medium (Bécard and Fortin, 1988) agar (1% w/v) containing 10⁻³ M GA₃, 10⁻² M uniconazole-P, or a mock solution (Bécard’s M medium).

**Construction of Binary Vectors**

The promoter sequences of GA20ox1 (2,533 bp), GA20ox2 (2,601 bp), and GA3ox1 (2,489 bp) were amplified by using Prime STAR GXL (Takara) and a thermal cycler (GeneAmp PCR system 9700; ABI). The amplified fragments were cloned into pENTR D/TOPO (pENTR D/TOPO cloning kit; Invitrogen). The coding sequence of the open reading frame was amplified by using PrimeSTAR GXL (Takara) and a thermal cycler (GeneAmp PCR system 9700; ABI). The amplified fragments were cloned into pENTR D/TOPO (pENTR D/TOPO cloning kit; Invitrogen). To create promoter-GUS fusions constructs, the open entry clones were converted into the destination vector pGKWPS7 (Kariimi et al., 2002) by using in vitro site-directed recombination (Gateway LR Clonase Enzyme mix; Invitrogen). The sequence of the *L. japonicus* GA1 homolog gene identifier, chm.0021.150.02 was retrieved from the *L. japonicus* genome database (miyakogusa.jp, assembly build 25; Sato et al., 2006). The open reading frame was amplified and cloned into pENTR D/TOPO as described above. Deletion of the DELLA domain of GA1 and integration of the GA1 promoter (2,536 bp) were performed by using PCR on the entry clones and ligation (DNA Ligation Kit Mighty Mix; Takara). The entry clones containing delta-GAI or GA1–delta-GAI were converted into the destination vector p35S-GW- GFP or p35S-GW-DSRed, respectively. The CaMV 35S promoter of p35S-GW-DSRed was removed by the destination vectors by Spel and HindIII digestion before the conversion to GA1–delta-GAI. The SbtM1 promoter-GUS and Delta-GAI–GUS constructs were described previously (Takeda et al., 2009, 2012). For coexpression of GOF-CCaMK and delta-GAI, SbtM1 promoter-GUS and the 35S–delta-GAI including the 35S–CCaMK1–2-NLS-NOS terminator were amplified by PCR (Prime STAR GXL; Takara) and the amplicons were ligated (DNA Ligation Kit Mighty Mix; Takara). The destination vectors without the Gateway cassette (p35S-GFP or p35S-DSRed) were used as vector controls. Primer sequences are listed in Supplemental Table S2.

**Hairy Root Transformation**

Transformation of *L. japonicus* was performed by using hairy root induction by *Agrobacterium rhizogenes* AR1193 as described by Takeda et al. (2013). Transgenic roots carrying GFP or DSRED cotransformation markers were selected by their fluorescence using a fluorescence stereomicroscope (SZX12; Olympus).

**Transcriptome Analysis by RNA-Seq**

Four weeks after inoculation with AM fungi, total RNA was extracted from seven to 10 roots (inoculated or not) by using the Plant RNA Reagent (Invirogen) and further purified by using the RNeasy Mini Kit and the RNeasy-free DNase Set (both from Qiagen). RNA quality was assessed with the RNA 6000 Nano Kit and the 2100 Bioanalyzer system (both Agilent Technologies). Before RNA-Seq analysis, the induction of the AM marker genes SbtM1 and PTA4 was confirmed in the RNA samples from roots inoculated with AM fungi (44%–88% colonization levels) by real-time qRT-PCR. RNA-Seq libraries were prepared from 500 µg of total RNA from three infected and three uninfected roots of *L. japonicus* versus *Ram1* or *Ram2* were versus RAM1 and RAM2. This indicates that these two pairs of genes are regulated by different branches of the symbiotic signaling pathway downstream of CCaMK and that GA signaling interferes with both branches. GA signaling or DELLA proteins are known to cross talk with various plant hormone signaling pathways (Achard et al., 2003; Fu and Harberd, 2003; Navarro et al., 2008; Hou et al., 2010; Gallego-Bartolomé et al., 2012). A recent study revealed that SLR1, the rice DELLA protein, interacts with the strigolactone receptor DWF414 (Nakamura et al., 2013). The interaction indicates that GA signaling may interfere with the strigolactone signaling pathway that influences AM fungal colonization (Yoshida et al., 2012). Thus, the GA effect on symbiosis observed here might be indirect and caused by GA affecting other hormonal signaling pathways. SLR1 also interacts with a RAM1 homolog through the GRAS family transcriptional regulator DELLA-INTERACTING PROTEIN1 (Yu et al., 2014). Such a protein-protein interaction may provide a direct link between GA and the symbiosis signaling pathway. These signaling pathways potentially influence the symbiosis signaling pathway and alter arbucular mycorrhiza development. Spatial and temporal changes in GA signaling had different effects on AM colonization (Fig. 3; Floss et al., 2013; Foo et al., 2013), and the pleiotropic effects of GA on arbucular mycorrhiza would be caused by differential interferences that depend on the levels of GA signaling.
biological replicates using the TruSeq RNA sample preparation kit (Illumina) according to the manufacturer’s instructions (Illumina adaptor indexes 1, 2, and 2 for the uninfected root samples and indexes 3, 8, and 9 for the infected root samples). The library quality was assessed with the Agilent DNA 1000 kit and the 2100 Bioanalyzer system. The libraries were sequenced (single-end sequencing, 100 bp) with the HiSeq 2000 sequencing system (Illumina). The uninfected sample (indexes 1 and 2) and the infected sample (indexes 3) were read in the same lane of the eight-lane HiSeq 2000 flow cell, and all other index samples were read in another lane. In three independent experiments, we obtained 10.7, 11.9, and 15.1 mega reads from the infected samples and 9.6, 9.7, and 12.3 mega reads from the uninfected samples. The acquired sequences were mapped on the genome of *L. japonicus* (Sato et al., 2008) by using TopHat software (Trapnell et al., 2009) with default parameters, except that maximum intron length was set at 10,000. The expression profiles were compared between the uninfected roots and the infected roots, and the arbuscular mycorrhiza-induced genes were identified by statistical analyses. Normalization of the mapped sequences was performed by the TMM-baySeq-TMM method (Hardcastle and Kelly, 2010; Robinson et al., 2010; Robinson and Oshlack, 2010; Kadota et al., 2012) implemented in R software (http://www.r-project.org/index.html) with a bootstrap number of 10,000.

**Real-Time qRT-PCR Analysis**

Real-time qRT-PCR analysis was performed by using the ReverTra Ace qPCR RT Kit and Thunderbird qPCR Mix (both from Toyobo) with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. Complementary DNA was synthesized from 500 ng of total RNA in a 20-μL reaction mixture. The complementary DNA (0.5 μL) was added to the real-time PCR mixture (20 μL). The primer sets are listed in Supplemental Table S2. Amplification conditions were as described by Takeda et al. (2013). Fold changes in expression were calculated by using the delta-delta cycle threshold method and normalized to the transcription level of *UBQUITIN* (Takeda et al., 2009). Average and SE values were calculated for three to five biological replicates.

**Quantification of Endogenous Plant Hormones**

The fresh weight of the roots with or without AM fungus inoculation was measured at 3 wai. The roots were frozen in liquid nitrogen and homogenized with Tissuelyser (Qiagen) and 5-mm zirconia beads. Quantification of plant hormones was performed by UPLC-ESI-qMS/MS using an ultra-performance liquid chromatograph coupled with a tandem quadrupole mass spectrometer system (Acquity UPLC System/XEVO-TQS; Waters) equipped with an electrospray interface, as described previously (Kojima et al., 2009).

**Histochemistry and Microscopy**

Transgenic roots carrying the promoter-GUS fusions were treated with GUS staining buffer (Takeda et al., 2009) at 37°C for 3 to 12 h. AM fungal structures in the host root were stained by the ink-stain method (Demchenko et al., 2004) or with WGA-Alexa Fluor 594 conjugate (Invitrogen; Harrison et al., 2002). Roots were heated in 5% (w/v) KOH at 95°C for 1 h (for ink staining) or 15 min (for WGA-Alexa Fluor staining) and washed three times with water. Then, for ink staining, the roots were heated in a solution containing 3% (v/v) black ink and 5% (v/v) acetic acid at 95°C for 20 min. For WGA-Alexa Fluor staining, the roots were immersed in phosphate-buffered saline containing WGA-Alexa Fluor 594 (1 μg mL⁻¹ final concentration) and kept at room temperature for at least 1 h. Calcofluor White (1 mg mL⁻¹) in phosphate-buffered saline was added after WGA-Alexa Fluor staining for plant and fungal cell wall staining. Bright-field and fluorescence microscopy was performed with stereomicroscopes (SZX12 and SZX16; Olympus), an upright microscope (BX50; Olympus) equipped with 10× (0.30 numerical aperture [NA]) and 20× (0.50 NA) dry objectives, and a digital camera system (DP73; Olympus). Z-stack images were acquired with a confocal microscope (A1; Nikon) equipped with a 20× (0.75 NA) dry objective.

**Quantification of AM Fungal Colonization**

The main root infected with AM fungi was stained with ink or WGA-Alexa Fluor 594, and the frequency of colonization (the intercellular fungal structures per unit of root length) was calculated by the magnified intersections method (McConigle et al., 1990; Takeda et al., 2009). The number of branched hyphae was counted in a 1-mm region from the tip of the most elongated hypha at 2 wai. The measurements were performed using the SZX12 or SZX16 stereomicroscope.

Raw RNA sequencing from this article can be found in the DNA Data Bank of Japan Sequence Read Archive (accession no. DRA002601).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Expression of the genes involved in GA biosynthesis and metabolism in uninfected control roots.

**Supplemental Figure S2.** Hyphal branching of AM fungi in the host roots.

**Supplemental Figure S3.** Numbers of branched hyphae in the host roots treated with GA₃, uniconazole-P, or both.

**Supplemental Figure S4.** Effect of GA or GA biosynthesis inhibitor on AM spore germination.

**Supplemental Figure S5.** Domain organization of *L. japonicus* GAI and overexpression phenotype of dominant-negative GAI.

**Supplemental Figure S6.** AM colonization of the roots carrying dominant-negative GAI.

**Supplemental Figure S7.** Arbuscular mycorrhiza-induced gene expression in the roots treated with GA₃.

**Supplemental Table S1.** Genes up- or down-regulated in the roots infected with *R. irregularis* (4 wai), identified by RNA-Seq analysis.

**Supplemental Table S2.** Sequences of PCR primers used in this study.

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**LITERATURE CITED**


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