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Plant Genes Related to Gibberellin Biosynthesis and Signaling Are Differentially Regulated during the Early Stages of AM Fungal Interactions

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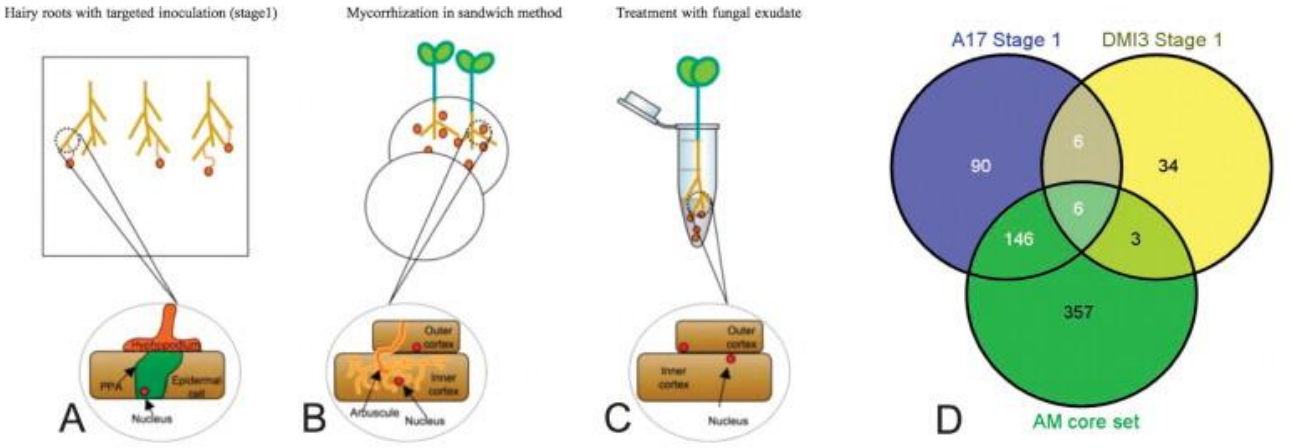
Dear Editor,

Phytohormones are essential regulators of plant development, but their role in the signaling processes between plants and fungi during arbuscular mycorrhizal (AM) establishment is far from being understood (Ludwig-Müller, 2010). AM colonization leads to extensive effects on host metabolism, as revealed by transcriptome studies of AM plants (Hogekamp et al., 2011). Some genes have been specified as an AM core set, since they are mycorrhizal-responsive, irrespective of the identity of the plant, of the fungus, and of the investigated organ. These data support the idea that, on colonization, plants activate a wide reprogramming of their major regulatory networks and argue that mobile factors of fungal or plant origin are involved in such generalized metabolic changes. In this context, hormones may be good candidates (Bonfante and Genre, 2010). However, the emerging picture of the interaction between phytohormones and AMs is very patchy, and information on gibberellin (GA) involvement is still more limited (García-Garrido et al., 2010). The role of GA during nodulation is instead known to control the nodulation signaling pathway (Ferguson et al., 2011).

Due to the overlap in signal transduction between *Rhizobium* symbiosis and AMs (Bonfante and Genre, 2010), a search was made for genes involved in GA synthesis and signaling in AMs, focusing on the early stages of the interaction. To reach this goal, we used Medicago GeneChips with RNA isolated from defined fragments of GFP-HDEL expressing transgenic roots contacted by fungal hyphopodia (see 'Supplemental Methods'). In this condition, the epidermal cells react by assembling a pre-penetration apparatus (PPA), which represents an early cytological marker of plant cells (Genre et al., 2005) and identifies epidermal cells about to be colonized by AM fungi. Since the so-called common SYM genes, which define a signaling pathway that mediates nodulation and mycorrhization, are known to closely control the early phases (Genre et al., 2005), transcriptome studies have also included a comparison with *M. truncatula MtDmi3* mutant roots. DMI3 is a plant calcium-calmodulin-dependent protein kinase (CCaMK) that plays a central role in the symbiotic signaling pathway. Plants mutated in the *dmi3-1* gene do not elicit PPA formation and do not establish the symbiosis (Genre et al., 2005).

Via hybridizations of *Medicago* Genechips of root fragments from wild-type (WT) and *MtDmi3* mutant enriched for early-contact stages (Figure 1A), 248 genes were identified as being at least twofold differentially regulated in WT roots at an FDR-corrected *P*-value of $P < 0.05$ (Figure 1D). The dataset is available from the Gene Expression Omnibus (accession number GSE34617, Supplemental Table 1). We found that 236 genes were exclusively up-regulated in WT roots following fungal contact, while 37 were activated in the *MtDmi3* mutant and 12 were jointly activated (Figure 1D). Six of these commonly activated genes were related to secondary metabolism or hormone signaling (Supplemental Figure 1). Although the isolated root fragments were enriched for early stages of AM interactions, arbuscule development could already commence. To rule out that transcription was related to arbuscule presence, we compared expression data with the core set of *M. truncatula* genes activated at 28 d post inoculation (dpi) during AM interaction (Hogekamp et al., 2011). These 'virtual subtractions' revealed that 96 *M. truncatula* genes were activated in WT roots during the early but not during the late AM stages, and 90 genes were expressed in an *MtDmi3*-dependent way (Figure 1D and Supplemental Table 2). This set of 90 genes was dominated by 29 genes related to secondary metabolism and hormone signaling (Supplemental Figure 1).



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Gene product (SAMS annotation)	NBCI Blastp annotation (ref_seq)	Arrays						RT-Real Time			
		28 dpi		Target inoculation				Target inoculation		Target inoculation	
		Gm myc	Gi myc	Gi.m Mt	p-value	Gi.m DMI3	p-value	Mt	SD	DMI3	SD
Gibberellin 3-beta-dioxygenase	Gibberellin 3-beta-dioxygenase (XP_003588693.1)	0,23	0,06	1,16	0,168	0,58	0,506	8,44	5,21	16,02	0,29
Gibberellin 2-beta-dioxygenase 7	Gibberellin 20 oxidase 1-like (XP_003528366.1)	-0,33	0,22	2,77	0,060	2,06	0,219	6,66	0,04	3,38	0,10
Ent-kaurenoic acid hydroxylase (KAO1) / cytochrome P450 88A3, putative (CYP88A3)	Ent-kaurenoic acid oxidase 2-like (XP_003546291.1)	0,89	0,38	1,37	0,329	1,52	0,335	0,20	0,09	0,84	0,35
Scarecrow-like transcription factor PAT1	Chitin-inducible gibberellin-responsive protein (XP_003617966.1)	-0,30	0,05	1,17	0,617	2,56	0,308	0,49	0,05	1,58	0,53
DELLA protein GAI	DELLA protein DWARF8-like (XP_003528281.1)	0,01	-0,77	1,32	0,138	1,03	0,289	0,16	0,17	0,29	0,11
Probabile Gibberellin receptor GID1L3	Probable carboxylesterase 15-like (XP_00352150)	0,12	0,15	-0,48	0,750	0,30	0,857	4,03	2,09	1,97	0,41
Probable gibberellin receptor GID1L2	Gibberellic acid receptor-b (XP_003591590.1)	-1,18	-0,23	1,88	0,347	2,21	0,327	0,04	0,01	0,21	0,04

Figure 1. The drawings (A-C) illustrate the setup developed to detect gene expression, while D-E summarize the main expression results.

(A)*M. truncatula* hairy roots inoculated with *Gi. margarita* spores at specific root sites. The fungus develops a hyphopodium and the epidermal cell reacts by producing the PPA.

(B) The sandwich method leads to a full colonization in 28d, allowing the formation of branched arbuscules inside cortical cells.

(C)*M. truncatula* seedlings treated with fungal exudates produced by germinating spores.

(D) Venn diagram summarizing the transcriptional responses of *M. truncatula* roots sampled at stage 1, showing the numbers of genes activated in different conditions: *M. truncatula* WT *versus Mtdmi3* mutant roots at fungal contact stage and *versus* the whole AM core set identified by Hogekamp et al. (2011) in fully mycorrhizal roots.

(E) The table lists the genes detected in the two array experiments and validated with RT-Real time. (i) *Medicago truncatula* at 28dpi with *G. mosseae* (Gm myc) and *G. intraradices* (Gi myc) (Hogekamp et al., 2011); and (ii) targeted inoculation of *M. truncatula* transformed roots with *Gi. margarita* (Gi.m) and sampled at hyphopodium contact, as well as of *M. truncatula MtDmi3* mutant transformed roots (Gi.m DMI3).

The array data revealed *M. truncatula* genes involved in the GA synthesis or signaling. Some of these are at the basis of isoprenoid metabolism, such as geranylgeranyl pyrophosphate synthase, which is a crucial enzyme for the synthesis of phytophormones and phytoene synthase that leads to phytoene synthesis (Supplemental Table 2). Since we were interested in detecting potential interactions between early plant/fungal contacts and GA, we looked for GA-related genes, taking into account both the tendency of gene expression induction in GeneChip experiment and information from annotation. Following this rationale, additional genes were detected coding for GA biosynthetic enzymes, and related to GA signaling (Figure 1E). Homologs of genes involved in the GA biosynthesis were already identified as mycorrhizal-responsive in tomato plants at the mature (50-dpi) phase of the interaction (García-Garrido et al., 2010). Instead, under our experimental conditions, these genes were slightly up-regulated in the fungal-contacted root fragments (Figure 1E) in WT and mutant genotypes, compared to the fully mycorrhizal roots.

The second group of genes related to gibberellin signaling included two putative gibberellin receptors, the negative regulator DELLA protein GAI and a Scarecrow-like transcription factor (Figure 1E). A member of this TF family has been described as a gene that promotes GA signaling in *Arabidopsis* by antagonizing the growth repressor DELLA (Zhang et al., 2011). It is known that the GA–GID1 receptor complex interacts with DELLA, leading to the DELLA proteolysis, de-repression, and transcription of GA-responsive genes (Yamaguchi, 2008). However, there is a lack of information on this signaling pathway during AM symbiosis establishment: our array experiment demonstrates that these genes are slightly up-regulated in the fungus-contacted roots, compared to whole mycorrhizal roots (Figure 1E).

The qRT–PCRs (Figure 1E) confirmed the up-regulation of the three genes involved in gibberellin synthesis in both WT and mutant roots upon fungal contact, while the genes involved in gibberellin signaling revealed a more heterogeneous pattern (Figure 1E). Even with some modulations, none of the genes seemed to be affected by the mutation in the *MtDMI3* gene, since the up-regulation trend

was maintained in both genotypes. We conclude that the gibberellin pathway is at least in part SYM-independent. The mutant genotype was therefore not included in the following experiments.

Gi. margarita is known to release bioactive molecules that are perceived by the host root cells (Bonfante and Genre, 2010). In order to test whether these released molecules have an effect on the gibberellin metabolism, *M. truncatula* seedlings were treated with *Gi. margarita* exudates for 5, 12, and 24 h (Figure 1C). When the results observed for the early stage were compared with those obtained after investigating the gene expression on fully *Gi. margarita* mycorrhizal roots at 28 dpi (Figure 1A, 1B, and 1E), the data revealed that gibberellin synthesis had already been activated by the fungal treatment (Supplemental Figure 2), peaked at the contact phase, and decreased during mycorrhization (Supplemental Figure 3), according to the initial array results. This picture corresponds well with the pattern shown by *GID1L3* and the DELLA protein, which were more up-regulated by the fungal factor treatment (Supplemental Figure 2). *GID1L3* was not expressed in mycorrhizal roots, unlike the DELLA protein, whose transcripts were up-regulated during the symbiotic phase (Supplemental Figure 3).

Transcriptomic data suggest that the endogenous GA may complex with the GA receptor during the early interaction phase, and interact with the negative DELLA protein regulator, thus leading to gibberellin activity. The low abundance of transcripts observed during the symbiosis of all the investigated genes with the exception of the DELLA protein could suggest a general repression of the gibberellin activity, which is possibly mitigated by the antagonistic role played by the Scarecrow gene (Zhang et al., 2011).

In conclusion, our transcriptome experiment has provided novel information on gene expression during the early plant–fungal contact; it offers a collection of genes activated in an *MtDmi3*-dependent way, thus being connected to the common SYM pathway, and specifies a limited number of genes that appeared to be SYM-independent. This dataset complements information already available for whole mycorrhizal roots (Hogekamp et al., 2011) and offers a novel source for candidate genes responding to early fungal signals and involved in secondary metabolism and hormone pathways.

The limited set of genes involved in GA metabolism turned out to be CCaMK-independent, but sensitive to signals originating from the symbiotic fungus and present in the exudate. Given that the bioactive molecules recently identified as LipoMyc CO have been shown to stimulate root branching (Maillet et al., 2011), potential novel roles may be hypothesized for GA concerning root development in the presence of mycorrhizal fungi. Taken as a whole, these observations are beginning to shed light on how complex networks may control the GA-mediated establishment of AM symbiosis and can provide a first response to the so-far unassigned role of this hormonal class.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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