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Seven *Lotus japonicus* Genes Required for Transcriptional Reprogramming of the Root during Fungal and Bacterial Symbiosis [W]

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A combined genetic and transcriptome analysis was performed to study the molecular basis of the arbuscular mycorrhiza (AM) symbiosis. By testing the AM phenotype of nodulation-impaired mutants and complementation analysis, we defined seven *Lotus japonicus* common symbiosis genes (*SYMRK*, *CASTOR*, *POLLUX*, *SYM3*, *SYM6*, *SYM15*, and *SYM24*) that are required for both fungal and bacterial entry into root epidermal or cortical cells. To describe the phenotype of these mutants at the molecular level, we screened for differentiating transcriptional responses of mutant and wild-type roots by large-scale gene expression profiling using cDNA-amplified fragment length polymorphism. Two percent of root transcripts was found to increase in abundance during AM development, from which a set of AM-regulated marker genes was established. A Ser-protease (*SbtS*) and a Cys-protease (*CysS*) were also activated during root nodule development. AM-induced transcriptional activation was abolished in roots carrying mutations in common symbiosis genes, suggesting a central position of these genes in a pathway leading to the transcriptional activation of downstream genes. By contrast, AM fungus-induced gene repression appeared to be unaffected in mutant backgrounds, which indicates the presence of additional independent signaling pathways.

INTRODUCTION

Legumes can form root endosymbioses with both phosphorus-acquiring arbuscular mycorrhizal (AM) fungi and nitrogen-fixing rhizobial bacteria. Although ~80% of all living land plants form AM (Read et al., 2000; Brundrett, 2002), the nitrogen-fixing root nodule symbiosis (RNS) with rhizobia is almost exclusively restricted to legumes. Signal exchange during the early stages of the bacterial interaction includes flavonoids secreted by the plant roots, which stimulate the biosynthesis of bacterial lipochito-oligosaccharides, the so-called Nod factors (NF). In turn, NF are perceived as symbiotic signals by the plant and induce root hair deformation, plant gene expression, and, depending on the host species, activation of cortical cells, leading to the formation of preinfection threads and/or nodule meristems (Oldroyd and Downie, 2004). Despite the importance of AM for plant nutrition, the molecular events that accompany this symbiosis are not well understood, partly because of the obligate biotrophic nature and the complex genetics of the fungal microsymbiont. For example, symbiotic signals from the fungus that induce plant gene expression at a distance have been detected but not characterized (Kosuta et al., 2003). However, the morphological description of the fungal infection process in the legume *Lotus japonicus* has revealed a series of successive steps that are under the genetic control of the plant (Bonfante et al., 2000; Novero et al., 2002; Demchenko et al., 2004; Parniske, 2004). Upon fungal contact, the anticlinal walls of two adjacent epidermal cells separate and allow the entry of the fungus into the resulting cleft. Genetic analysis suggested that this epidermal opening response is an active process that involves the *L. japonicus SYM15* gene (Demchenko et al., 2004). The fungus subsequently enters either one of the neighboring epidermal cells or the

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underlying hypodermal cells and continues to grow intracellularly through one or two more cell layers before it exits a plant cell to explore the extracellular spaces of the root cortex. Arbuscules are highly branched intracellular fungal structures that are formed upon fungal entry into cortical cells. Expression patterns of plant transporter genes suggest that at least part of the nutrient exchange between the symbiotic partners occurs within arbusculated plant cells (Rausch et al., 2001; Harrison et al., 2002; Paszkowski et al., 2002).

In legumes, the genetic programs for fungal and bacterial symbiosis partially overlap; legume genes have been identified that are required for the establishment of both AM and RNS (Hirsch et al., 2001; Marsh and Schultze, 2001; Stougaard, 2001; Parniske, 2004) and are referred to as the common *SYM* genes (Kistner and Parniske, 2002). Common symbiosis mutants were identified in *Medicago sativa*, *Pisum sativum*, *L. japonicus*, *Medicago truncatula*, *Phaseolus vulgaris*, *Vicia faba*, and *Melilotus alba* (Duc et al., 1989; Bradbury et al., 1991; Sagan et al., 1995; Shirtliffe and Vessey, 1996; Schauser et al., 1998; Szczyglowski et al., 1998; Wegel et al., 1998; Catoira et al., 2000; Senoo et al., 2000; Kawaguchi et al., 2002; Lum et al., 2002).

Cloning of a series of key genes required for symbiosis led to the identification of the Nod factor receptor kinase genes *NFR1* and *NFR5* (Madsen et al., 2003; Radutoiu et al., 2003). Moreover, four common *SYM* genes have been cloned, and their predicted protein products carry the hallmarks of signal transduction components. *L. japonicus SYMRK* (previously *SYM2* [Schauser et al., 1998] and *SYM21* [Szczyglowski et al., 1998]) encodes a receptor kinase (Stracke et al., 2002) the activity of which is regulated by phosphorylation (Yoshida and Parniske, 2005).

Orthologous receptor kinases have been identified from *M. sativa* (NORK), *M. truncatula* (DMI2), and *P. sativum* (SYM19) (Endre et al., 2002; Stracke et al., 2002). Phenotypic analysis of mutant plants has established a role of this receptor kinase in a pathway leading from the perception of the NF signal to the activation of symbiosis-related gene expression (Schneider et al., 1999; Catoira et al., 2000; Stracke et al., 2002). The *L. japonicus* genes *CASTOR* (previously *SYM4* [Schauser et al., 1998], *SYM22* [Szczyglowski et al., 1998], and *SYM71* [Kawaguchi et al., 2002]) and *POLLUX* (previously *SYM23* [Szczyglowski et al., 1998] and *SYM86* [Imaizumi-Anraku et al., 2004]) encode closely related proteins that are predicted ion channels (Imaizumi-Anraku et al., 2004). The Medicago *DMI1* gene (Ane et al., 2004) is probably a *POLLUX* ortholog (Imaizumi-Anraku et al., 2004), whereas a *CASTOR* ortholog from Medicago has not been described. Medicago *DMI3* encodes a calcium and calmodulin-dependent protein kinase (Levy et al., 2004; Mitra et al., 2004a). These predictions, together with a detailed phenotypic analysis of the corresponding mutants (Catoira et al., 2000; Imaizumi-Anraku et al., 2004; Mitra et al., 2004b), strongly suggest a role for these genes in signaling downstream of NF perception.

Analysis of calcium spiking, one of the earliest responses of root hair cells to NF application (Ehrhardt et al., 1996), placed the pea and Medicago orthologs of *SYMRK* (*PsSYM19*, *NORK*, and *DMI2* [Endre et al., 2002; Stracke et al., 2002]) as well as *CASTOR* and *POLLUX* upstream of this response (Wais et al., 2000; Walker et al., 2000; Harris et al., 2003; Imaizumi-Anraku et al., 2004), indicating that these genes act at a similar hierarchical level very early in the symbiotic signaling process. By contrast, *M. truncatula* DMI3 has been placed downstream of the calcium-spiking response (Wais et al., 2000) and has structural features such as EF hands and a calmodulin binding domain that conceptually allow it to interpret the calcium oscillations and convert them to a phosphorylation reaction as a readout (Levy et al., 2004; Mitra et al., 2004a).

Plant genes that are transcriptionally regulated in response to symbiotic stimuli can serve as useful markers to monitor the activity of signaling pathways. There are three classes of these genes, nodulins, mycorrhizins, and symbiosins, that are activated in response to a rhizobial signal, an AM fungal signal, or by either stimulus, respectively. From the large number of nodulins (van Kammen, 1984) and early nodulin (ENOD) genes (Kouchi et al., 2004), a subset has been found to be also activated in response to inoculation with AM fungi (Frühling et al., 1997; van Rhijn et al., 1997; Albrecht et al., 1998; Journet et al., 2001; Manthey et al., 2004). In *M. truncatula*, DMI1, DMI2, and DMI3 have been placed upstream of symbiosis-induced gene activation, as accumulation of >30 transcripts, including *MtENOD11*, *MtENOD12*, and *MtRIP1*, by either rhizobial inoculation or NF application is impaired in *dmi1*, *dmi2*, and *dmi3* mutant backgrounds (Catoira et al., 2000; Mitra et al., 2004b).

To further dissect the plant's common program for fungal and bacterial symbiosis, we searched for additional common *SYM* mutants of *L. japonicus*. We present the outcome of complementation studies between mutants originating from three independent mutagenesis experiments, which resulted in the identification of seven common *SYM* genes. To characterize their symbiotic phenotypes in detail, we studied the morphology of the root responses to fungal and bacterial symbionts. Furthermore, a large-scale gene expression profiling experiment in *L. japonicus* was performed to obtain insight into the changes in the root transcriptome during AM formation and to compare the transcriptome of wild-type roots with that of mutants affected in common *SYM* genes. We established a central role of the common *SYM* genes in the transcriptional reprogramming of the root during AM and RNS. Moreover, AM-induced gene repression patterns provided evidence for the existence of signal perception and transduction that occur independently of common *SYM* genes.

RESULTS

Seven *L. japonicus* Genes Are Required for RNS and AM

Chemical (ethyl methanesulfonate [EMS]), T-DNA, or transposon-tagging mutagenesis in the legume *L. japonicus* yielded a large number of nodulation-impaired mutants originating from different laboratories (Schauser et al., 1998; Szczyglowski et al., 1998; Perry et al., 2003). We tested these nodulation mutants for their ability to form symbiosis with the AM fungus, *Glomus intraradices*, and identified a group of mutants that were also defective in AM formation. Because of their common impairment in both root symbioses, we refer to these lines as common *SYM* mutants. Complementation analyses of mutants described by Schauser et al. (1998), Szczyglowski et al. (1998), and Novero

et al. (2002) were performed, and seven complementation groups were established. The results of complementation and phenotypic analyses are summarized in Table 1. The symbiotic phenotypes in all complementation groups segregated as recessive monogenic traits (data not shown). The mycorrhiza and nodulation phenotypes completely cosegregated in F2 progeny of crosses to the mapping parents *L. japonicus* ecotypes MG-20 and Funakura or *L. filicaulis*, providing evidence that mutations in single genes are responsible for the observed deficiencies in both symbioses.

Fungal Infection Is Aborted in the Outer Cell Layers

In earlier reports, the consequences of mutations in the *L. japonicus* common *SYM* genes *SYMRK*, *CASTOR*, and *SYM15* for the AM symbiosis were described at the cytological level (Bonfante et al., 2000; Novero et al., 2002; Demchenko et al., 2004). The fact that mutations in *POLLUX*, *SYM3*, *SYM24*, or *SYM30* cause an AM defect has been stated previously, but with little or no phenotypic detail (Szczyglowski et al., 1998; Wegel et al., 1998; Imaizumi-Anraku et al., 2004). Here, we analyzed fungal infection of the outer root cell layers of representative common *sym* mutants (Table 1, Figure 1). Fungal hyphae were observed to penetrate between two epidermal cells in the wild type (Figure 1A) and mutants affected in the *SYMRK*, *CASTOR*, *POLLUX*, *SYM3*, *SYM6*, or *SYM24* genes (Figures 1C to 1H; data not shown), suggesting that the epidermal opening response was unaffected. Furthermore, fungal hyphae were found to continue to explore extracellular spaces between outer cell layers, or sometimes even to penetrate into cells. These exploratory hyphae often show balloon-like swellings or other deformations, which are typically associated with infection arrest and were not observed on wild type roots (Table 1). Interestingly, such exploratory hyphae leading to hyphal deformation below the root surface are not observed on roots of the *sym15-2* mutant (Figure 1B). At the morphological level, the fungus is blocked on *sym15-2* roots at an earlier stage than on any of the other mutants. We conclude that *sym15-2* is impaired in the epidermal opening response as described by Demchenko et al. (2004), whereas the other tested common *sym* mutants are not.

Mutations in Common *SYM* Genes Affect Arbuscule Formation with Different Severity

A limited ability of certain common symbiosis mutants of *L. japonicus* to form arbuscules has been described previously (Wegel et al., 1998; Novero et al., 2002; Demchenko et al., 2004). We analyzed the AM phenotypes of the common *sym* mutants at two different time points to evaluate their potential for, and to determine the timing of, arbuscule formation (Table 1). After 2 weeks of cocultivation with the AM fungus, abundant arbuscules

Table 1. Allelism and Phenotypes of Common Symbiosis Mutants Originating from Independent Mutagenesis Experiments

		Mesorhizobium loti		*Glomus intraradices*		
					Arbuscule Formation	
Common *SYM* Gene (Previous Designation)	Mutant Allele Tested (Line Description)	Infection Threads	Nodule Meristem	Balloon-Like Swellings in Outer Cell Layers	2 wai	6 to 8 wai
	Gifu wild type	+	+	−	+	++
SYMRK (*SYM2*, *SYM21*)	*symRK-3* (cac41.5)[a]	−[b]	−	+	−	+
	symRK-7 (EMS61)[a]	−[b]	−	+	−	+
CASTOR (*SYM4*, *SYM22*, *SYM71*)	*castor-1* (282-227)[c]	−[d]	−	+	+	+
	castor-2 (EMS1749)[c]	−[d]	−	+	−	−
	castor-4 (EMS46)	−[d]	−	+	−	+
POLLUX (*SYM23*, *SYM86*)	*pollux-1* (EMS70)	−[d]	−	+	−	+
	pollux-2 (EMS167)	−[d]	−	+	−	+
SYM3	*sym3-1* (5371-22)	−	−[e]	+	−	+
	sym3-3 (EMS247)	−	−[e]	+	−	+
SYM6	*sym6-1* (10512.9)	−	+	n.d.	n.d.	n.d.
	sym6-3 (EMS126)	−	+	+	−	+[f]
SYM15	*sym15-2* (cac57.3)[g]	−	−	−	−	−
SYM24	*sym24-1* (EMS76)	−	−[e]	+	−	+

Mutant lines with the prefix EMS were identified after EMS mutagenesis by Szczyglowski et al. (1998), with the exception of EMS1749, which was generated and identified by J.K. Webb (Novero et al., 2002). The remaining lines resulted from an attempt to generate tagged mutants through T-DNA transformation (Schauser et al., 1998). n.d., not determined; wai, weeks after inoculation.

[a] AM phenotypes observed here for *symRK* mutants confirmed cytological observations by Wegel et al. (1998) and Demchenko et al. (2004).

[b] Absence of infection threads has been described previously for *symRK* mutants (Stracke et al., 2002).

[c] AM phenotypes oberved here for *castor* mutants EMS1749 and 282-227 confirmed previous cytological observations using *G. margarita* as a fungal symbiont (Bonfante et al., 2000; Novero et al., 2002).

[d] Absence of infection threads has been described previously for *castor* and *pollux* mutants (Bonfante et al., 2000; Imaizumi-Anraku et al., 2004).

[e] *sym3* and *sym24-1* mutants formed small ineffective nodules at a low frequency of less than one nodule per plant.

[f] Only one arbuscule was observed in >30 root systems inspected.

[g] The AM phenotype observed here for the *sym15-2* mutant cac57.3 confirmed cytological observations by Demchenko et al. (2004).

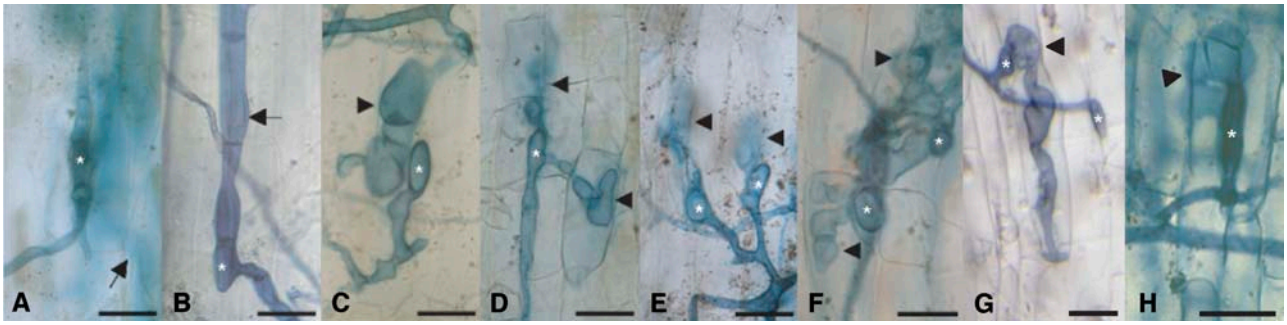

Figure 1. AM Infection on the Wild Type and Symbiotic Mutants.

Photographs of cleared *L. japonicus* roots stained with ink were taken after 6 weeks of cocultivation with *G. intraradices* in chive nurse pot systems. Bars = 25 μm.

(A) B-129 Gifu wild type. *G. intraradices* has formed an appressorium (asterisk) and entered the root between the longitudinal walls of two epidermal cells. Fungal hyphae (arrow) are directed toward the inner cortex, out of the focal plane.

(B) *sym15-2*. The fungus has formed an appressorium (asterisk) on the groove between two epidermal cells. The hyphae (arrow) failed to enter the root between epidermal cell walls but grew extraradically along the border of two epidermal cell files.

(C) *sym3-1*. The fungus has formed an appressorium (asterisk) and entered the root between the longitudinal walls of two epidermal cells. Hyphal growth into the inner cortex did not occur, and the infection process was aborted between the epidermal and exodermal cell layers (arrowhead). This is a typical example of the balloon-like swelling of fungal hyphae (arrowhead) frequently observed in this phenotypic class of mutants (Table 1).

(D) *pollux-1*. The infection process was as described for **(C)**. One hyphal branch of the hypha has penetrated an epidermal cell, where the infection aborted (bottom arrowhead).

(E) *sym24*. The infection process was as described for **(C)**.

(F) *sym6-3*. Two appressoria (asterisks) in close vicinity to each other between epidermal cells. Fungal hyphae originating from these appressoria branched and entered epidermal cells, where the infection process aborted (arrowheads).

(G) *castor-2*. The infection process was as described for **(C)**. A balloon-like hyphal swelling is indicated by the arrowhead.

(H) *symRK-2*. The infection process was as described for **(C)**. A balloon-like hyphal swelling is indicated by the arrowhead.

were present in roots of the wild type but not in the symbiotic mutants. The only exception was a mutant carrying the weak *castor-1* allele. At later time points, between 6 and 8 weeks of cocultivation with the AM fungus, rare successful infection events of the mutant root cortex by the fungus had occurred and arbuscules could be observed with variable frequency in the root system of mutants affected in the *POLLUX*, *SYM3*, or *SYM24* genes (Table 1). However, such delayed colonization of the root cortex frequently did not yield arbuscules in all inspected *symRK* mutants, in *sym3-1*, in *pollux-1*, and in the *sym24-1* mutant. This differs significantly from the wild-type colonization pattern, in which arbuscules form without major delay after fungal hyphae reach the root cortex. Arbuscules were not observed in roots of >100 *castor-2* and *sym15-2* mutant individuals, and only a single arbuscule was observed upon inspection of >30 root systems of *sym6-3* (Table 1).

Common *SYM* Genes Are Required for Infection Thread Initiation and Early Stages of Nodule Development

Nodules did not form on roots of *castor*, *pollux*, *symRK*, and *sym15-2* mutants and were severely reduced in development on *sym6* mutants (Schauser et al., 1998; Szczyglowski et al., 1998). Occasionally, nodules developed on *sym24* mutant roots at a frequency of approximately one nodule per seven root systems. Also on *sym3*, inefficient nodulation was observed at a low frequency (Table 1). We studied the ability of the mutants to form infection threads using a derivative of *M. loti* strain R7A that constitutively expresses the β-galactosidase reporter gene. Whereas infection threads could be clearly detected in wild-type root hairs, this was not the case in root hairs of most of the mutants listed in Table 1. Exceptions were root hairs of mutants in the *SYM3* gene, on which infection threads were observed but at a low frequency.

cDNA-Amplified Fragment Length Polymorphism Transcriptome Analysis Demonstrates That Plant Gene Activation during AM Depends on *CASTOR* and *SYM15*

To dissect the phenotypes of common symbiosis mutants at the molecular level, we performed transcriptional expression profiling of wild-type versus mutant roots. The aim was to establish a set of symbiosis-regulated marker genes that can be used to interrogate different mutant backgrounds to refine our picture of the signaling pathways leading to AM symbiosis. We chose the cDNA-amplified fragment length polymorphism (cDNA-AFLP) approach because it allows direct side-by-side comparison of transcript patterns across multiple samples and has the potential to visualize transcripts expressed at low levels (Bachem et al., 1996). Furthermore, it does not require prior sequence information or investment in array technology. Transcript profiles of *L. japonicus* uninoculated wild-type roots were compared with those of roots inoculated with either the AM fungus *G. intraradices* or *M. loti* to identify mycorrhizins and symbiosins.

Approximately 7000 cDNA amplification products were inspected, of which 344 were differentially represented. The

majority of differential fragments were exclusively present or strongly enhanced in wild-type mycorrhized roots (Figures 2A and 2F; see Supplemental Table 1 online). A second group of fragments represented genes with a nodulin-like expression pattern (Figure 2E; see Supplemental Table 1 online). Only six fragments were enhanced in both symbiotic interactions (potential symbiosins) (Figure 2F; see Supplemental Table 1 online).

Consistent with the presence of hyphae in fungus-inoculated root samples, several sequenced fragments were likely to be derived from the fungus *G. intraradices*, as judged from sequence similarity to either fungal DNA or fungal protein sequences. For 10 fragments, the nonplant origin was confirmed experimentally (see Supplemental Table 2 online). At least 50% of the AM-specific cDNA-AFLP fragments were considered to be of plant origin because they exhibited strong sequence similarity to plant ESTs, primarily from *L. japonicus*, *M. truncatula*, or *Glycine max*. Because ~4% of all displayed cDNA-AFLP fragments were specific to roots infected with AM fungus, the cDNA-AFLP analysis suggests that at least 2% of the plant root transcriptome changes during AM development.

The cDNA-AFLP analysis included *castor-2* and *sym15-2* mutant roots inoculated with *G. intraradices* (Figure 2) to visualize the effects of common *sym* mutations on root transcript profiles. These two reference lines were chosen because arbuscule development was never observed in their roots (Table 1) (Bonfante et al., 2000; Novero et al., 2002; Demchenko et al., 2004). Thus, misleading false-positive expression patterns resulting from rare successful breakthrough events could be avoided. The vast majority of plant cDNA fragments that were induced by AM in wild-type samples were not induced in either of the two mutant lines. Although 40 fragments were enhanced both in the wild type and in one or both mutants (Figures 2B and 2C; see

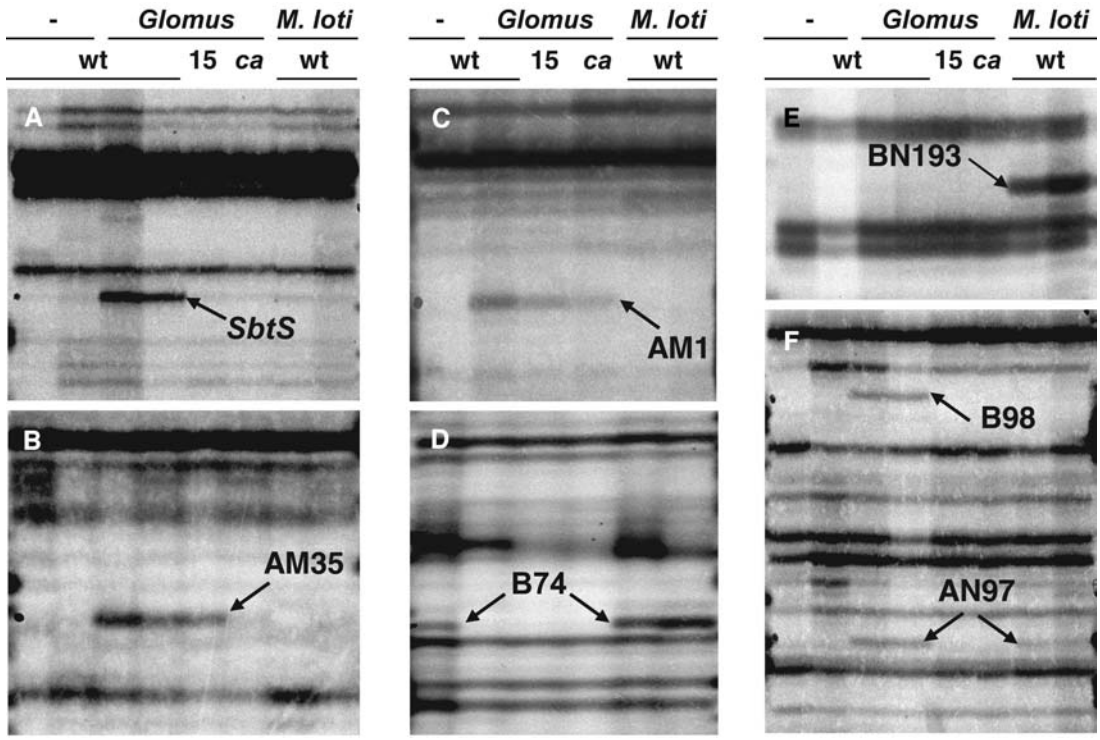

Figure 2. Transcript Profiling of Symbiotic Roots by cDNA-AFLP.

Autoradiographs of AFLP gels of cDNA samples prepared from 3-week-old *L. japonicus* roots grown in the absence of symbiont (−), grown for 2 weeks in the presence of *G. intraradices* (*Glomus*), or from nodulated roots (*M. loti*). Genetic backgrounds include wild-type *L. japonicus* Gifu (wt), mutant *sym15-2* (15), and mutant *castor-2* (ca). Duplicate samples for *G. intraradices*–inoculated and noninoculated wild-type roots in **(A)**, **(B)**, **(E)**, and **(F)** are from two independent experiments to monitor the reproducibility of transcript profiles. Samples from nodulated roots in each panel were taken, from left to right, 14 or 21 d after inoculation of 6-d-old seedlings with *M. loti*.

(A) *SbtS* cDNA fragment enhanced in mycorrhized wild-type roots but not in symbiotic mutants or nodulated roots. The expression of this gene was found subsequently to be transiently induced upon *M. loti* inoculation (see Figure 4A).

(B) cDNA fragment AM35 was detected in wild-type and *sym15-2* mutant roots after *G. intraradices* inoculation.

(C) cDNA fragment AM1 was detected in wild-type and mutant roots after *G. intraradices* inoculation.

(D) B74 represents a glutathione *S*-transferase gene repressed upon *G. intraradices* inoculation in both wild-type and symbiotic mutant roots.

(E) BN193 was expressed only in nodulated roots (potential nodulin).

(F) B98 was expressed exclusively in mycorrhized wild-type roots (potential mycorrhizin). AN97 was expressed in wild-type roots upon inoculation with either *G. intraradices* and *M. loti* (potential symbiosin).

Supplemental Table 1 online), many of them turned out to be of fungal origin. For some plant-derived fragments (e.g., S259; see Supplemental Table 1 online), further investigations confirmed the differential expression pattern but suggested that expression was also influenced by factors other than symbiosis. Therefore, the activation of the majority of plant genes during AM is *CASTOR*- and *SYM15*-dependent.

Because the *sym15-2* and *castor-2* mutants differ in their AM phenotype, one might expect this difference to be reflected by differential gene activation. Indeed, a small number of plant-derived cDNA fragments were enhanced during AM but specifically absent from either *castor-2* or *sym15-2* root samples (see Supplemental Table 1 online). Two of these fragments represented two *L. japonicus* GDSL motif–containing lipase genes (Symbiosis-Induced Lipase-Like genes [*SILLI1a* and *SILLI1b*; see Supplemental Table 1 online]). Both genes were activated in the wild type and *castor-2* but not in *sym15-2* roots upon infection with the AM fungus, and this pattern was confirmed by RT-PCR. Sequence analysis of large-insert genomic clones revealed the presence of a lipase gene family in *L. japonicus* in which at least eight members are arrayed in tandem. The expression of highly similar gene family members was found to be dependent on plant age, and transcripts were also detected in leaves (data not shown). Interestingly, enhanced expression was also observed in nodulated roots (data not shown). Because of this complexity, we did not subject the lipase gene family to a refined expression analysis.

Novel Marker Genes for Symbiotic Root Development

We used semiquantitative RT-PCR with gene-specific primers as an independent method to verify the expression pattern of a subset of differentially displayed cDNA-AFLP fragments. Figure 3 shows a time course of transcript accumulation during AM development for a subset of genes exhibiting a robust and reproducible AM-responsive expression pattern. Most of these genes were analyzed further by determining their corresponding full-length cDNA as well as their genomic sequences (see Supplemental Table 3 online). The latter was done within the context of the *L. japonicus* genome sequencing project.

The abundance of a fungal cDNA fragment from a predicted aspartyl protease gene (*Gi131*) was found to correlate with the extent of root colonization by the fungus (data not shown). Therefore, *Gi131* was used as a marker for the degree of fungal colonization (Figures 3, 4A, and 4C; see Supplemental Table 2 online).

Three Protease Genes and a Germin-Like Protein Gene Are Activated Early during AM Symbiosis

Among the AM-induced genes, *SbtM* and *SbtS* show strong similarity to Ser-protease–encoding genes of the subtilase superfamily (Siezen and Leunissen, 1997). *L. japonicus* mycorrhiza-subtilase (*SbtM*) transcripts (Figure 3) were not detected in uninoculated roots. Their steady state levels increased with the degree of mycorrhizal colonization, as reflected by the accumulation of the fungal *Gi131* RT-PCR product (Figure 3) and observed after trypan blue staining of part of the root samples

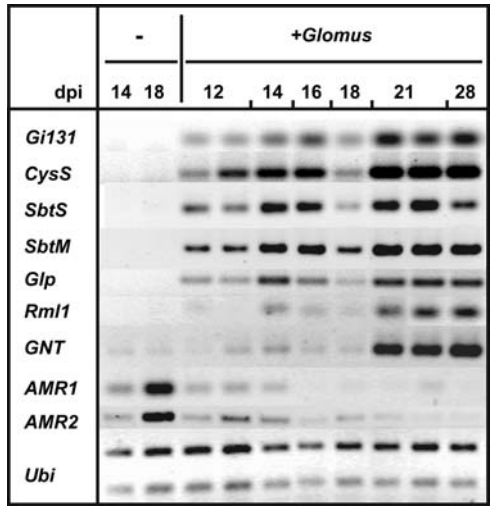

Figure 3. Time Course of AM-Regulated Gene Expression.

Transcript levels in roots uninoculated (−) or inoculated with *G. intraradices* (+*Glomus*) harvested at different days postinoculation (dpi) were analyzed by RT-PCR. Induction of the *L. japonicus* genes *CysS*, *SbtS*, *SbtM*, *Glp*, *Rml1*, and *GNT*, and repression of *AMR1* and *AMR2*. A cDNA fragment of a fungal aspartate protease gene (*Gi131*) was amplified as a measure of the degree of colonization by the AM fungus. A fragment of a constitutively expressed *L. japonicus* polyubiquitin gene (*Ubi*) was amplified as a control for the amount of RNA template in the reactions. The first arbuscules were already observed at 6 dpi, and the degree of arbuscular colonization increased until 6 weeks after inoculation. In the experiment shown, the 18-dpi sample exhibited an atypically low degree of fungal colonization, as detected by trypan blue staining (data not shown) and the low abundance of fungal transcript *Gi131*. Both induction and repression of AM-regulated plant genes were lower in this sample, indicating that the level of gene regulation was a function of the degree of fungal colonization and not of plant age. Two independent samples for the 12- and 21-dpi harvest were included to document biological variation.

(data not shown). *SbtM* gene induction was observed exclusively in wild-type roots inoculated with *G. intraradices* but not in other plant tissues or under different growth conditions tested. In four independent experiments, *SbtM* transcript levels in uninoculated roots were at or below the detection limit of RT-PCR. *SbtM* expression was not detected in roots upon NF treatment or *M. loti* inoculation (Figure 4A; data not shown), nor was it apparent during phosphate starvation or in powdery mildew–infected leaves (data not shown). No *SbtM* transcript could be detected in mutant roots affected in either of the common *SYM* genes with or without *G. intraradices* at 7, 14, or 21 d after inoculation (Figure 4; data not shown). Therefore, activation of *SbtM* is specific for AM and requires the function of *SYMRK*, *CASTOR*, *POLLUX*, *SYM3*, *SYM6*, *SYM15*, and *SYM24*.

The second subtilase gene, *SbtS*, was also strongly upregulated after inoculation with the AM fungus (Figure 3), and this upregulation was significantly attenuated in mutants (Figures 4A to 4C). Induction was also observed in *M. loti*–inoculated roots (Figure 4A). Therefore, the gene was named *SbtS* for *L. japonicus*

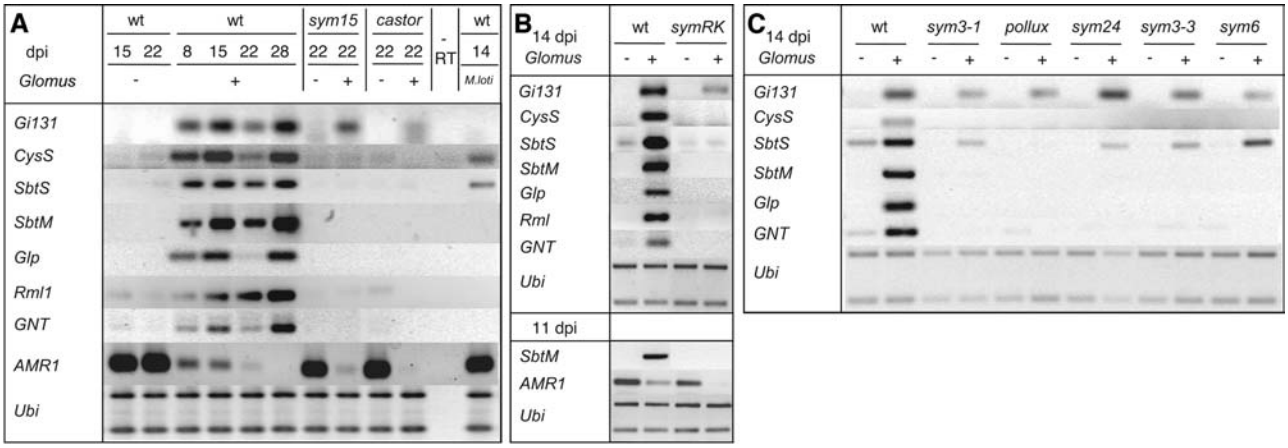

Figure 4. Expression of AM-Regulated Genes in Symbiotic Mutants of *L. japonicus*.

RT-PCR analysis of transcript levels in roots of the wild type (wt), *sym15-2* (*sym15*), and *castor-2* (*castor*) **(A)**; roots of the wild type and *symRK-3* (*symRK*) **(B)**; and roots of the wild type, *sym3-1* (*sym3-1*) *pollux-1* (*pollux*), *sym24* (*sym24*), *sym3-3* (*sym3-3*), and *sym6-3* (*sym6*) **(C)**. Noninoculated roots (−) and roots inoculated with *G. intraradices* (+) or *M. loti* (*M. loti*) were harvested at different days after inoculation (dpi) for RNA extraction. −RT indicates control PCR without prior reverse transcription, using as template RNA of mycorrhized wild-type roots at 3 weeks after inoculation. A *L. japonicus* polyubiquitin gene (*Ubi*) was amplified as a control for equal amounts of template between samples.

symbiosis subtilase. Neither *SbtM* nor *SbtS* transcripts were detected in *L. japonicus* leaves or stems (data not shown).

Two independent cDNA-AFLP fragments with sequence similarity to Cys-proteases were found to be induced during AM. RT-PCR experiments using primers matching one of the fragments revealed gene induction as early as 8 d after inoculation with the AM fungus and also in nodulated roots (*CysS*; Figures 3 and 4). Amplification of cDNA fragments by 5′ and 3′ rapid amplification of cDNA ends (RACE) as well as genomic sequencing of BAC clones revealed the presence of a tandem array of several very closely related family members in *L. japonicus* (data not shown). The expression pattern of individual members of this family has yet to be determined. In addition, mRNA corresponding to a cDNA fragment with sequence similarity to germin-like protein (*Glp*) genes also accumulated early during AM (Figures 3 and 4A).

Induction of *SbtS* Expression by NF or Rhizobia Is Strongly Attenuated in *symRK*, *castor*, and *sym15* Mutants

A time course of *SbtS* activation after inoculation with *M. loti* strain R7A or treatment with NF was analyzed (Figure 5A). Induced expression was detected at the earliest time points investigated, 1 h after NF treatment and 6 h after bacterial inoculation. Expression remained strong during the first days after inoculation but was lower in roots with 2-week-old nodules and could not be detected at 7 weeks after inoculation. This transient expression pattern is probably responsible for the lack of a detectable *SbtS* cDNA-AFLP fragment 2 and 3 weeks after inoculation with *M. loti* (Figure 2A). The full response to bacteria is dependent on NF, as *M. loti* strain R7AC2, a bacterial *nodC* mutant unable to produce NF, did not induce the gene expression to a similar extent as wild-type bacteria (Figures 5A and 5B). Full activation of *SbtS* during the interaction with rhizobia depends on the function of *CASTOR*, *SYM15*, and *SYMRK*, because only a weak and transient activation occurred in roots of the corresponding symbiotic mutants (Figure 5B). Such weak and transient induction was reproducibly observed in three different mutants representing *symRK-2*, *symRK-3*, and *symRK-7* alleles (Figure 5B; data not shown). A *L. japonicus* leghemoglobin gene induced early upon NF or *M. loti* inoculation (Stracke et al., 2002) was included as a reference marker (Figure 5B). The induction of this gene by NF or *M. loti* was compromised in plants homozygous for the *castor-2*, *sym15-2*, or *symRK-3* allele (Figure 5B).

Rml1, a Remorin Gene, and *GNT*, a *N*-Acetylglucosaminyltransferase Gene, Are Activated Later during AM Development

L. japonicus RML1 is homologous with remorin from potato (*Solanum tuberosum*), a protein that is attached to the plasma membrane, binds oligogalacturonids, endogenous elicitors of plant wound responses (reviewed in León et al., 2001), and becomes phosphorylated in a ligand-dependent manner (Reymond et al., 1996). The *GNT* gene encodes a protein with 80% similarity to *N*-acetylglucosaminyltransferases, which are involved in glycoprotein modification in the Golgi (β-1,4-mannosyl-glycoprotein β-1,4-*N*-acetylglucosaminyltransferase). The induction of *Rml1* and *GNT* upon fungal inoculation (Figure 3) is *CASTOR*-, *SYM15*-, and *SYMRK*-dependent (Figures 4A and 4B). The expression of *Rml1* and *GNT* was delayed relative to the other AM-induced genes (Figure 3). Transcript levels of *Rml1* were not increased above the basal level at 7 or 8 d after inoculation (Figure 4A) but only at 12 to 14 d after inoculation and became more pronounced at later time points (Figures 3 and 4A). By contrast, both subtilase genes were clearly upregulated at the earliest time points investigated (7 and 8 d after inoculation; Figure 4A; data not shown). Given these differences in the timing of expression, the subtilase genes could be

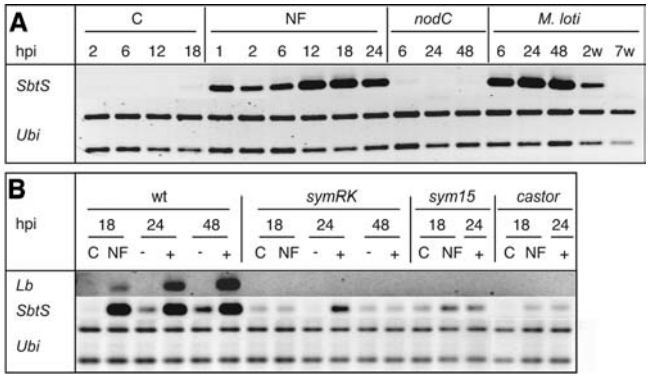

Figure 5. Early Expression of *SbtS* and *Lb* in Response to NF or *M. loti*.

Transcript abundance analyzed by RT-PCR in *L. japonicus* roots treated with *M. loti* NF, mock-treated (C), or inoculated with *M. loti* strain wild-type R7A (*M. loti*, +) or R7AC2 carrying a *nodC* mutation (*nodC*, −). Roots were harvested for RNA extraction hours (hpi) or 2 or 7 weeks (2w, 7w) after inoculation. A *L. japonicus* polyubiquitin gene (*Ubi*) was amplified to control for equal amounts of template between samples.
(A) Time course of gene expression in wild-type plants.
(B) Expression pattern in the symbiotic mutants *symRK-3* (*symRK*), *sym15-2* (*sym15*), and *castor-2* (*castor*).

categorized as early and *Rml1* and *GNT* as late AM-induced genes. It will be interesting to correlate the expression of these late and early genes with the development of specific symbiotic structures.

Early and Sustained Activation of the *Cbp1* Promoter during AM

β-Glucuronidase (GUS) activity in roots of a transgenic line harboring a *Cbp1* promoter:GUS fusion (T90; Webb et al., 2000) was previously found to be induced rapidly upon treatment with *M. loti* (Webb et al., 2000) (Figure 6A). Because one of our goals was to identify useful molecular markers, we tested the responsiveness of this promoter during AM development. The T90 line carrying Pro_{Cbp1}:*GUS* was inoculated with *G. intraradices*, and GUS activity was detected after 4 d, the earliest time point analyzed (data not shown). GUS activity was also observed in cells not in direct contact with fungal hyphae (Figures 6E and 6F), and expression persisted during later stages of AM development (Figures 6E to 6H). Strong GUS activity was detected not only in arbuscule-containing cells but also in cells of the pericycle and vascular tissue that are never infected by fungal hyphae (Figure 6H). Our results demonstrate that the *Cbp1* promoter is activated early during AM infection and that direct fungal contact with a host cell is not required for Pro_{Cbp1}:*GUS* activation within that cell. The T90 line was crossed to *castor-2* and *sym15-2*, and individual F2 mutants homozygous for the Pro_{Cbp1}:*GUS* insertion were identified using an insertion-specific PCR marker (see Methods). F3 progeny of the *castor* and *sym15* mutants carrying the Pro_{Cbp1}:*GUS* insertion did not show any GUS staining upon either fungal or rhizobial inoculation (Figures 6B, 6C, 6I, and 6J). These results indicate that *CASTOR* and *SYM15* are required for Pro_{Cbp1}:*GUS* activation upon stimulation with either the fungal or the bacterial microsymbiont.

Downregulation of *AMR1* Gene Expression by Fungal Inoculation Occurs in Common *sym* Mutants

The largest group of differentially displayed cDNA-AFLP fragments represents plant genes that are upregulated in mycorrhized wild-type *L. japonicus* roots but not in the symbiotic mutants *castor-2* and *sym15-2* (Figure 2A). However, an additional expression pattern indicative of transcriptional regulation that occurred independently of the *CASTOR* or *SYM15* gene was detected.

Interestingly, for all nine of the AM-repressed cDNA-AFLP fragments, the pattern suggested repressed expression in both wild-type and mutant roots after inoculation with *G. intraradices* (Figure 2D). Two of the fragments, corresponding to genes *AMR1* and *AMR2* (for AM repressed), were chosen for analysis in RT-PCR experiments, and the mycorrhiza-dependent repression was confirmed (Figure 3). Database searches did not reveal a function for *AMR1*, whereas *AMR2* is probably a pseudogene. Although the full-length cDNA of *AMR2* exhibited sequence similarity to Ser-protease inhibitor genes of the Serpin family, the cDNA suffers from a frameshift within three nucleotides after the start codon, which leads to its translation into a protein completely unrelated to serpins. The open reading frame comprises only 402 bp, less than the first half of the 1.3-kb cDNA. Curiously, the same frameshift was found in ecotypes Gifu and MG-20 and the distantly related species *L. filicaulis*, indicating that this frameshift predated speciation (data not shown).

AMR1 repression after infection with the AM fungus occurred to the same extent in roots of the wild type and both *castor-2* and *sym15* mutants (Figure 4A). Downregulation was also observed in roots of a *symRK* mutant (Figure 4C), although it was less pronounced in an independent experiment (data not shown). A similar expression pattern had been observed for a gene of *M. truncatula*, *Mt4*, which is downregulated by AM fungi in wild-type roots of both *M. truncatula* and *M. sativa* and in roots of the *M. sativa nork* mutant MN-1008 (Burleigh and Harrison, 1997). Interestingly, *Mt4* is also repressed under moderate and high phosphate conditions (Burleigh and Harrison, 1997, 1998). In *L. japonicus* roots, a moderate level of phosphate fertilization of 0.2 mM compared with the 0.01 mM used in controls had no detectable effect on *AMR1* expression (data not shown). *AMR1* transcript abundance exhibited a diurnal rhythm, with transcript levels being highest in the morning (data not shown).

DISCUSSION

Seven *L. japonicus* Genes Are Required for Intracellular Infection by Fungal and Bacterial Symbionts

Here, we show that in *L. japonicus* at least seven genetic loci control early steps of both fungal and bacterial symbiosis. The microscopic analysis of their mutant phenotypes indicated that these genes are involved in the successful intracellular infection by microsymbionts. During the interaction with *M. loti*, infection threads were not formed. Although mutants in *SYMRK*, *CASTOR*, *POLLUX*, *SYM3*, *SYM15*, and *SYM24* do not form nodules or form nodules at very low frequency (Table 1), small nodule-like structures were observed on mutants defective in the *SYM6*

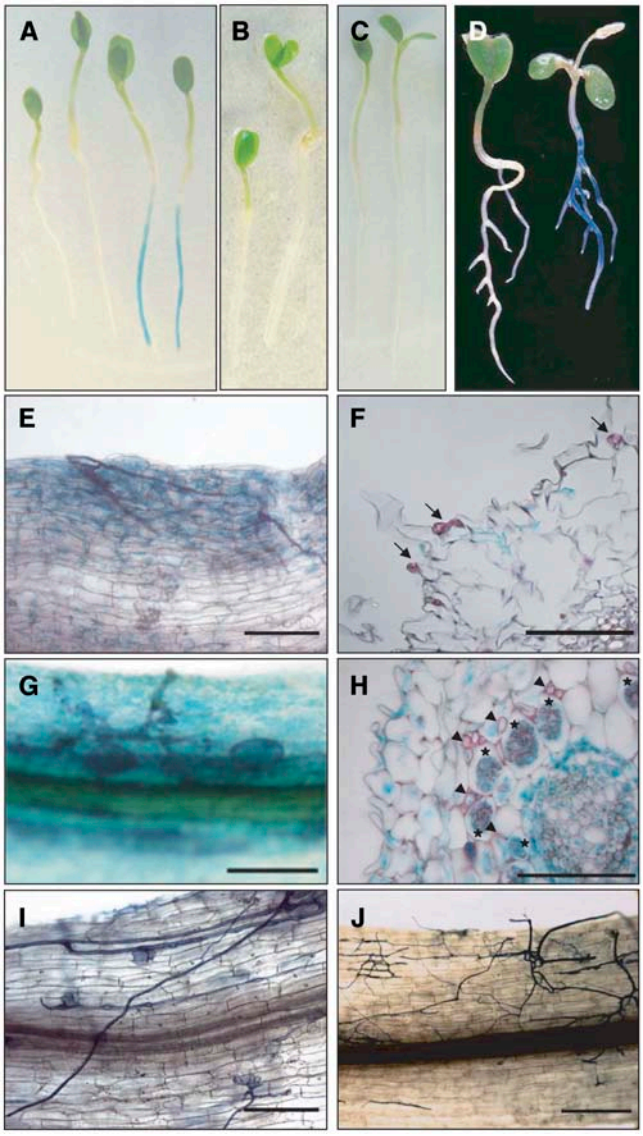

Figure 6. Early Symbiotic Activation of Pro_{Cbp1}:*GUS* in AM and RNS Is Abolished in *castor-2* and *sym15-2* Mutant Backgrounds.

Expression and histochemical localization of Pro_{Cbp1}:*GUS* in roots of transgenic line T90 (**[A]** and **[D]** to **[H]**) and F3 progeny of *castor-2* × T90 (**[B]** and **[I]**) and *sym15-2* × T90 (**[C]** and **[J]**) after inoculation with *M. loti* (**[A]** to **[C]**) or *G. intraradices* (**[D]** to **[J]**). In **(E)**, **(G)**, **(I)**, and **(J)**, root samples were double stained with black ink for fungal structures and with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid for GUS activity. Bars = 200 μm (**[E]** and **[H]** to **[J]**) and 100 μm (**[F]** and **[G]**).

(A] to **(C)** GUS activity was detected 24 h after inoculation with *M. loti* in 5-d-old T90 roots (**[A]**, right) but not in T90 noninoculated control roots (**[A]**, left) or in inoculated roots of *castor-2* × T90 **(B)** and *sym15-2* × T90 **(C)**.

(D) GUS activity in T90 roots after 2 weeks of cocultivation with *G. intraradices* (right) but not in noninoculated control roots (left).

(E) and **(F)** GUS activity in T90 roots after 1 week of cocultivation with *G. intraradices*. Expression was analyzed in whole roots **(E)** and semithin cross sections **(F)**. GUS activity was found in epidermal and exodermal cells associated with extraradical hyphae (arrows).

gene, indicating that this gene is not required for the induction of cell divisions leading to nodule primordium formation (Schauser et al., 1998). It is unlikely that this finding is attributable to allele leakiness, because the *sym6-3* mutation results in a very strong AM phenotype (Table 1). This mutant, therefore, uncouples nodule initiation from infection, which further supports the view that the genetic overlap between the two symbioses is at the level of intracellular infection.

On all common *sym* mutant roots listed in Table 1, the infection by AM fungi is aborted either before cell entry or within the first penetrated cells. Hyphae are either blocked at the surface (*sym15-2*) or continue to explore extracellular spaces between outer cell layers or sometimes even penetrate into cells. These exploratory hyphae often show balloon-like swellings or other deformations that are a diagnostic feature of aborted infections. This fact clearly indicates the requirement of the common symbiosis genes for fungal intracellular infection of the outer cell layers.

Old roots of *symRK* mutants, harboring likely null alleles such as transposon insertions and truncations, developed infrequent but structurally intact arbuscules, indicating that this gene is not absolutely required for the formation of these symbiotic structures (Stracke et al., 2002; Demchenko et al., 2004). By contrast, the *CASTOR* and *SYM15* genes are indispensable for arbuscule development (Novero et al., 2002; Demchenko et al., 2004). A role for the *SYM6* gene during arbuscule development is likely because arbuscules were detected at a very low frequency in roots of the *sym6-3* mutant (Table 1). The *pollux*, *sym3*, and *sym24* mutants form arbuscules with a similar delay as *symRK* mutants (Table 1). However, intraradical infection patches were observed in these mutants that were not associated with arbuscules but only with extracellular hyphae. Therefore, it is possible that these genes make incremental contributions to arbuscule development. Significant phenotypic variation between alleles has been observed in different *castor* and *sym15* mutants (Novero et al., 2002; Demchenko et al., 2004). Therefore, careful side-by-side comparisons of null alleles of *POLLUX*, *SYM3*, and *SYM24* will be required to determine their precise role during later stages of AM development.

Large-Scale cDNA-AFLP Profiling Reveals Major Transcriptional Reprogramming during AM Symbiosis

To obtain insights into the role of the common *SYM* genes in fungal AM symbiosis, we performed transcript profiling by cDNA-AFLP, which has the potential to display a large proportion of an mRNA population and therefore is suitable for genome-scale analysis (Bachem et al., 1996). Our cDNA-AFLP profiling

(G) and **(H)** GUS activity in T90 roots after 4 weeks of cocultivation with *G. intraradices*. Expression was analyzed in whole roots **(G)** and semithin cross sections **(H)**. GUS stain localized to various cell types including arbuscule (asterisks) and non-arbuscule-containing cells. The arrowheads indicate intercellular hyphae.

(I) and **(J)** Extraradical hyphae and appressoria formation on roots of *castor-2* × T90 **(I)** and *sym*15-2 × T90 **(J)**. No GUS activity was detected.

experiment revealed that the plant root undergoes substantial transcriptional reprogramming during AM development; ~2% of all plant transcripts were upregulated, and a small number of transcripts were downregulated.

We compared the sequences obtained from our cDNA-AFLP screen with those described by other laboratories to be AM-regulated. Four *L. japonicus* AM-induced fragments showed similarity to *M. truncatula* sequences from a cDNA library enriched for AM-specific root transcripts (MtGIMs; Wulf et al., 2003). These exhibited sequence similarity to a Cys-protease, a germin-like protein, a glutathione *S*-transferase, and an *N*-acetylglucosaminyltransferase. A cDNA with sequence similarity to genes for Cys-rich antifungal proteins (S212; see Supplemental Table 1 online) was detected in nodulated *L. japonicus* roots, whereas an MtGIM clone with high sequence similarity to this fragment was found in mycorrhized roots of *M. truncatula* (Wulf et al., 2003; Manthey et al., 2004). Transcript profiling in Medicago using cDNA arrays resulted in several AM-regulated genes (Liu et al., 2003; Manthey et al., 2004). Three genes identified by Liu et al. (2003) showed sequence similarity to *Lotus* cDNA-AFLP fragments: a Cys-protease, a glutathione *S*-transferase, and a subtilase. *MtENOD8.1*, a GDSL motif–containing lipase for which a high expression during nodulation was known, was found to increase expression also in AM roots of *M. truncatula* (Manthey et al., 2004). Like the *L. japonicus* GDSL lipase genes identified in this study (see Supplemental Table 1 online), *MtENOD8.1* is part of a gene cluster. Brechenmacher et al. (2004) and Manthey et al. (2004) identified a putative peptidyl-prolyl *cis-trans* isomerase gene as upregulated in mycorrhized roots. A *L. japonicus* cDNA-AFLP fragment with high sequence similarity (B135, S302; see Supplemental Table 1 online) was decreased 2 weeks after inoculation with AM fungi (data not shown).

The conserved activation in both Lotus and Medicago supports the idea that these genes might be of functional relevance during AM. However, because most of these genes are members of larger gene families, it remains to be established whether orthologous (i.e., functionally identical) gene pairs have been identified.

Common *SYM* Genes Are Required for the Activation of Most AM-Regulated Genes

Not a single plant gene could be identified that was reproducibly activated in a truly symbiosis-specific manner independent of the common *SYM* genes. Because of the scale of the cDNA-AFLP experiment, only a single time point was analyzed. Thus, it is possible that some transcripts were not detected at all or were missed because of a transient expression pattern, as exemplified by *SbtS* induction during nodulation (Figures 2A and 4A). However, the cumulative evidence provided by the substantial number of fragments inspected strongly suggests that the sustained transcriptional activation of the majority of genes in response to the AM fungus *G. intraradices* depends on the function of *CASTOR* and *SYM15* (Figures 2 and 4). This qualitative result from the cDNA-AFLP analysis was confirmed by a detailed RT-PCR analysis of a series of newly identified marker genes (Figure 4) and was extended to include the remaining common *SYM* genes. We conclude that the common *SYM* genes tested are key components of the transcriptional reprogramming of the roots during AM symbiosis.

This global effect of common *SYM* mutations on AM-activated transcription is intriguing given the complex expression patterns of AM-induced genes. The analysis of promoter–reporter fusions and in situ hybridization has revealed that different AM-induced genes are activated in different cell types and at different stages of AM development (Harrison, 1996; Chabaud et al., 2002; Harrison et al., 2002; Doll et al., 2003; Liu et al., 2003). There are several possible mechanisms through which gene expression could be affected in the mutants. The common SYM proteins could be involved directly in a signal transduction process leading to transcriptional gene activation. This is particularly likely for genes such as *Cbp1* because the *Cbp1* promoter:GUS fusion was activated early during infection and also induced in epidermal cells at a distance from fungal hyphae. However, some AM-responsive genes, such as phosphate and hexose transporter genes that are involved in arbuscule physiology, are activated only after fungal penetration into the root tissue (Harrison, 1996; Harrison et al., 2002). As mutations in the common *SYM* genes lead to an early arrest of fungal infection attempts at the root surface, the lack of activation of late genes could be an indirect consequence of a lack of arbuscule development. Therefore, it is possible that a direct defect in signaling in combination with the defect of AM development gives rise to the observed global lack of gene activation in the mutant lines analyzed.

Transcripts of *PsENOD5* and *PsENOD12A* do not accumulate in the interaction of roots of pea *Pssym8* mutants with *Gigaspora margarita* (Albrecht et al., 1998). Likewise, in *M. truncatula*, the AM fungus–induced transcriptional activation of several genes was not observed in a *dmi3* mutant background (Weidmann et al., 2004). Although these studies involved only individual mutants, the results can be taken as a first indication that the global effect of common *sym* mutants on transcriptional activation observed here in the *L. japonicus*/*G. intraradices* system may also take place at a similar scale in other legumes.

A role of common *SYM* genes in signal transduction is further supported by their mutant phenotypes in response to rhizobial signals. Its induction within 1 h or less upon NF treatment places *SbtS* among the earliest *ENOD* genes known to date, together with *MtRIP1* and *MtENOD12* (Pichon et al., 1992; Ramu et al., 2002). Also, the *Cbp1* promoter responds rapidly to *M. loti* (Webb et al., 2000) (data not shown). Because of the impairment of both *SbtS* and *Cbp1* responses in symbiotic mutants, we conclude that the corresponding common *SYM* genes have a role in the earliest stages of NF-mediated signaling.

Signal Transduction Independent of Common *SYM* Genes

Signaling through the common *SYM* genes is usually portrayed as a single pathway, mostly because the currently available tools are not sufficient for a more refined placement. Gene activation by rhizobia or AM fungi depends on common *SYM* genes, but a single linear pathway does not encompass the specific activation of nodulins and mycorrhizins in only one of the symbioses. Clearly, additional parallel pathways or a signaling

network are likely to mediate the development of nodulation and AM. At present, calcium spiking is the only molecular marker that distinguishes between common *sym* mutants. Therefore, one aim of this study was to use transcriptional gene regulation as a readout to decipher the activity of different signaling pathways and to identify regulons that require only a subset or none of the common *SYM* genes for their regulation. Indeed, we found that all AM-repressed cDNA-AFLP fragments (including *AMR1*) in our transcript profiling experiment were also repressed in *castor* and *sym15* mutant roots, providing clear evidence for the existence of signal transduction pathways, leading from the perception of the fungus by the plant to transcriptional gene regulation, that are independent of the common *SYM* genes.

METHODS

Biological Material and Growth Conditions

All plant mutant lines used were derived from *Lotus japonicus* ecotype B-129 Gifu, which is referred to as ''wild type'' throughout this study. The Pro_{Cbp1}*:GUS* fusion in line T90 was generated through a promoter-trapping experiment (Webb et al., 2000).

For inoculation of *L. japonicus* with *Mesorhizobium loti* R7A (Sullivan et al., 2002) or strain R7AC2 (*nodC*::Tn*5* mutant; J.T. Sullivan and C.W. Ronson, personal communication) and NF treatment, growth conditions and inoculation procedures were as described (Stracke et al., 2002).

Inoculation of seedlings with *Glomus intraradices* (strain deposited in the Bank of European Glomales as BEG195) was essentially as described (Wegel et al., 1998), except that plants were grown in expanded clay particles (Biosorb; PDI Agrochemical, Essex, UK), watered daily, and treated once per week with half-strength Hoagland solution (Hoagland and Arnold, 1950) with phosphate content reduced to 0.1 mM. Novel mutants were grown side by side with the already described mutants to allow direct comparison and to exclude the possible influence of different experimental conditions on their respective phenotypes.

Root Sectioning and Staining

Ink staining and sectioning of roots were done essentially as described (Demchenko et al., 2004). After staining for GUS activity, roots were cut into 5-mm-long pieces and fixed in 4% formaldehyde and 0.25% glutaraldehyde overnight at 4°C. Roots were washed for 1 h in double-distilled water, dehydrated in a graded ethanol series, equilibrated in Histo-Clear (National Diagnostics, Atlanta, GA), and embedded in Paraplast Plus (Sigma-Aldrich, St. Louis, MO). Serial sections (10 μm) were cut with a rotary microtome, attached to SuperFrost Plus slides (Roth, Karlsruhe, Germany), and deparaffinized in Histo-Clear. The tissue sections were rehydrated in a graded ethanol series, equilibrated in double-distilled water, and incubated in Schiff's reagent for 2 h for Feulgen staining (Demchenko et al., 2004). Then, sections were washed 3×15 min with SO_3/water (Demchenko et al., 2004) and 2×5 min with double-distilled water. Sections were dehydrated in a graded ethanol series, equilibrated in Histo-Clear, and mounted with Eukitt (Kindler, Freiburg, Germany).

cDNA-AFLP

For cDNA-AFLP, RNA was prepared from *L. japonicus* roots as described (Goormachtig et al., 1995). Of the extracted total RNA, 50 μg was used for poly(A)$^+$ extraction on Dyna beads (Dynal, Oslo, Norway) according to the manufacturer's instructions and was then subjected to cDNA synthesis. The cDNA-AFLP analysis was performed as described (Durrant et al., 2000) using 69 AFLP primer combinations. The ^{33}P-labeled cDNA fragments were separated on 6% polyacrylamide gels and visualized by autoradiography. DNA sequences of 205 reamplified cDNA-AFLP fragments were determined either by direct sequencing or after cloning.

RNA Extraction and RT-PCR

For RNA extraction (except for the cDNA-AFLP; see above), *L. japonicus* root material was ground in liquid nitrogen, thoroughly mixed with 500 μL of prewarmed extraction buffer (Chang et al., 1993), and extracted twice with 1 volume of phenol:chloroform:isoamyl alcohol (125:24:1, pH 4.7; Sigma-Aldrich). The aqueous supernatant was precipitated with LiCl at a final concentration of 3 M for 4 to 12 h at 4°C. After centrifugation, the pellet was washed with 75% ethanol, dried, and dissolved in water. Contaminating genomic DNA was removed by 30 min of incubation with 1 unit of DNaseI (Amersham Biosciences, Uppsala, Sweden) per microgram of RNA at 37°C in the presence of 10 units of SUPERasIn (Ambion, Austin, TX), followed by phenol:chloroform:isoamyl alcohol extraction and precipitation with sodium acetate:ethanol. Pellets were washed and dissolved in RNA-Secure resuspension solution (Ambion) at 60°C, and 1 μg of total RNA was used for first-strand cDNA synthesis.

Reverse transcription and PCR were performed as described (Stracke et al., 2002). For a control of equal amounts of cDNA template within the different samples, cDNA fragments of a *L. japonicus* polyubiquitin gene (AW719307) were amplified (Stracke et al., 2002). A reaction using RNA template without RT was routinely included as a control for the presence of genomic DNA contamination.

5′ RACE PCRs were performed using the FirstChoice RLM RACE kit (Ambion), according to the manufacturer's instructions. 3′ RACE was performed using either this kit or primer 10,011 (see Supplemental Table 4 online) as a reverse primer on oligo(dT)-primed cDNA. PCR conditions and primer sequences are listed in Supplemental Tables 3 and 4 online.

To confirm the origin of fungal cDNA fragments, RNA was extracted from chive (*Allium schoenoprasum*) roots infected with the same fungal isolate and subjected to cDNA synthesis as described above. In cases in which sequence-identical fragments could be amplified from cDNA samples from infected Lotus and chive roots, the fragment was considered to be of fungal origin (see Supplemental Table 2 online).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AP004549, AP004985, AP006863, AP006864, AP006865, AP006866, AP006147, AP006148, AJ609244 to AJ609269, and DN652254 to DN652405.

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