Evidence for the Involvement in Nodulation of the Two Small Putative Regulatory Peptide-Encoding Genes *MtRALFL1* and *MtDVL1*

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Nod factors are key bacterial signaling molecules regulating the symbiotic interaction between bacteria known as rhizobia and leguminous plants. Studying plant host genes whose expression is affected by Nod factors has given insights into early symbiotic signaling and development. Here, we used a double supernodulating mutant line that shows increased sensitivity to Nod factors to study the Nod factor-regulated transcriptome. Using microarrays containing more than 16,000 70-mer oligonucleotide probes, we identified 643 Nod-factor-regulated genes, including 225 new Nod-factor-upregulated genes encoding many potential regulators. Among the genes found to be Nod factor upregulated, we identified and characterized *MtRALFL1* and *MtDVL1*, which code for two small putative peptide regulators of 135 and 53 amino acids, respectively. Expression analysis confirmed that these genes are upregulated during initial phases of nodulation. Overexpression of *MtRALFL1* and *MtDVL1* in *Medicago truncatula* roots resulted in a marked reduction in the number of nodules formed and in a strong increase in the number of aborted infection threads. In addition, abnormal nodule development was observed when *MtRALFL1* was overexpressed. This work provides evidence for the involvement of new putative small-peptide regulators during nodulation.

Additional keywords: rhizobial infection.

Leguminous plants have the capacity to establish symbiotic interactions with specific soil bacteria that are collectively known as rhizobia. This interaction leads to the formation of new organs on the roots of host plants, called nodules, inside which atmospheric nitrogen is reduced for the benefit of the plant. This interaction is initiated by a molecular dialogue between the two symbiotic partners. Host plants excrete flavonoids that trigger bacteria to produce lipochitooligosaccharidic signals called Nod factors (NF) that are specifically perceived and transduced by the host plant (Geurts et al. 2005; Oldroyd

and Downie 2004; Stacey et al. 2006). Successful nodule development requires both the initiation of sequential cell divisions, first in the root pericycle and then in the inner cortical layers, leading to the formation of a nodule primordium, and the subsequent infection of the plant root by symbiotic bacteria via tubular structures called infection threads.

In the absence of rhizobia, purified NF have been shown to trigger many of the known early symbiotic responses, including specific calcium oscillations (Ehrhardt et al. 1992), and the expression of some of the so-called early nodulin genes such as *ENOD2*, *-11*, *-20*, *-40*, *NIN*, *RIP1*, and *MtANN1* (D'Haeze and Holsters 2002). Depending on the species considered, purified NF can, in addition, lead to different degrees of cortical cell activation (Niwa et al. 2001; Timmers et al. 1999; Truchet et al. 1991; van Brussel et al. 1992).

The first global survey of NF-affected genes was performed by Mitra and colleagues (2004) using Affymetrix microarrays representing 9,935 *Medicago truncatula* tentative consensus sequences (TC). These authors demonstrated that, of the 46 genes upregulated by *Sinorhizobium meliloti*, 40 were also induced by NF treatment. During previous studies of the NF-regulated transcriptome, we were limited by the difficulty of detecting weakly expressed genes or genes expressed in discrete subsets of cells responding to NF treatments (Godiard et al. 2007).

In *M. truncatula*, supernodulating mutants belonging to two complementation groups were originally described as showing more intense or sustained root infection and nodulation upon inoculation with *S. meliloti*: the autoregulation of nodulation (AON)-defective *sun* alleles (Penmetsa et al. 2003; Sagan et al. 1995, 1998; Schnabel et al. 2005) and the ethylene-insensitive mutant *sickle* (Oldroyd et al. 2001; Penmetsa and Cook 1997). Consequently, we decided to explore the transcriptional response to NF in *M. truncatula* using a double supernodulating mutant line to increase the detection sensitivity.

There are many reports demonstrating the importance of small regulatory peptides and secreted peptide hormones during developmental processes and defense mechanisms as shown, for example, for systemins, phytoalexins, clavata, and clavata-like peptides (Matsubayashi and Sakagami 2006; Ryan et al. 2002). Although growing evidence suggest that the establishment of the legume–*Rhizobium* spp. symbiosis leading to nodule initiation and development is regulated via classical phytohormones such as ethylene, auxin, and cytokinin (Hirsch and Fang 1994; Mulder et al. 2005; Oldroyd 2007) and, more recently, jasmonic and abscisic acids (Bright et al. 2005; Liang et al. 2007; Sun et al. 2006), few reports have

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demonstrated the involvement of small peptides in the regulation of nodulation. ENOD40 plays a central role during nodulation as an active mRNA (Campalans et al. 2004; Crespi et al. 1994). However, the two small conserved peptides it encodes may also have roles during nodule development (Rohrig et al. 2002; van de Sande et al. 1996). A large family of nodule-specific cysteine-rich (NCR) proteins that might act as antimicrobial defensins or diffusible signaling molecules assuring cell-to-cell communication has also been described (Graham et al. 2004; Mergaert et al. 2003; Silverstein et al. 2007). Recently, Oelkers and colleagues published an interesting study of the CLE peptide family in *Medicago* spp. and the potential role of two of them on root meristem development; however, nodulation was not examined (Oelkers et al. 2008).

Therefore, among the large collection of new NF-activated and NF-downregulated genes we identified in this study, we were intrigued to find two genes, *MtRALFL1* and *MtDVL1*, encoding small peptides proposed to play regulatory roles during plant development (Matsubayashi and Sakagami 2006). The rapid alkalization factor (RALF) was first identified during purification of systemins as a 49-amino-acid (aa) peptide that causes rapid medium alkalization but no defense responses at nanomolar concentrations in tobacco cell cultures (Pearce et

al. 2001) and has been suggested to act as a regulator of root growth and development (Wu et al. 2007). The *Devil* (*DVL*) gene family, also called *rotundifolia* (*ROT*), was discovered in two different screens for fruit and leaf shape mutants within activation-tagged mutant populations of *Arabidopsis thaliana* (Narita et al. 2004; Wen et al. 2004) and has been proposed to control polar cell proliferation in lateral organs.

Expression analysis of *MtRALFL1* and *MtDVL1*, presented in this study, suggest that these genes are upregulated by NF treatments and during initial phases of nodulation. In addition, overexpression studies suggest that these genes might impact on symbiotic rhizobial infection and possibly also nodule development (in the case of *MtRALFL1*).

RESULTS

The double supernodulating mutant *sun-2 sickle* as a tool to study NF responses.

In a search for plant lines with enhanced responses to NF treatments, we decided to test supernodulating mutants. We anticipated that combining the *sun-2* (Sagan et al. 1995) and *sickle* (Penmetsa and Cook 1997) mutant alleles in a single plant line would result in enhanced symbiotic responses, including

Table 1. Selection of genes predicted by microarray analysis to be upregulated by Nod factor (NF) treatments^a

Gene category ^c	MtGI8	MENS	NF/CF ^b		Gene name	Annotation
			6 h	48 h		
Markers	TC95529	MtC20218	3.45	3.25	<i>MtAnn1</i>	Annexin
	TC95776	MtD22261	...	2.20	<i>MtAnn2</i>	Annexin
	TC94515	MtC00043	1.89	...	<i>MtEnod40</i>	Enod40
	TC107261	MtC30514	...	1.92	<i>MtRip1</i>	Peroxidase
	TC102163	MtD27802	...	2.34	...	MtN1-like
	TC103530	MtD03150	...	1.89	...	MtN6-like protein
	TC96179	MtD02963	...	5.93	<i>MtN21</i>	MtN21
	TC109877	MtD06265	...	2.08	...	MtN21-like protein
	TC107654	MtC10311	2.75	...	<i>MtN24</i>	MtN24
	TC106859	MtC10163	...	2.20	...	Nonsymbiotic leghemoglobin 1
	TC110699	MtC40073	...	2.93	...	Symbiosome membrane nodulin
	TC108837	MtD02322	...	1.92	...	AP2 /ERF TF
	TC99463	MtC40098	...	1.87	<i>Mtern1</i>	AP2 /ERF TF
	TC95828	MtC10387	...	1.93	...	AP2 /ERF TF
TF	TC102833	MtD02676	...	1.74	...	AP2 /ERF TF
	TC103387	MtD04443	1.96	...	...	TF B3
	TC97132	MtD01395	...	1.81	...	bHLH TF
	TC95981	MtC10582	...	3.80	<i>MtHAP2-1</i>	CCAAT-binding B TF
	TC95764	MtD05991	...	2.56	...	Homeodomain leucine zipper protein
	TC102505	MtC50581	1.87	1.80	...	HD-ZIP factor
	AJ847976	MtC60288	...	1.82	...	HvB12D homolog
	TC102745	MtD00849	...	1.89	...	Myb TF
	TC108959	MtD02812	...	2.09	...	Myb (SHAQKYF class) TF
	TC99867	MtD06702	...	1.84	...	Myb (SHAQKYF class) TF
	TC96646	MtD05611	...	1.82	...	Myb (SHAQKYF class) TF
	TC98218	MtD05521	...	1.85	...	Zn finger protein
	TC96938	MtD03349	...	1.78	...	Leucine-rich repeat protein
	TC98446	MtC61331	...	2.05	...	Zn finger protein (C2H2 type)
Signal	TC96874	MtC50559	...	1.96	...	Putative BRI1-associated receptor kinase
	TC108221	MtD24088	...	1.83	...	Serine/threonine-protein kinase
	TC95903	MtC60470	...	2.44	...	Serine/threonine protein kinase
	TC110793	MtC61806	...	1.99	...	Putative cyclin dependent kinase
	TC98972	MtD06512	2.69	2.16	<i>MtLyk8</i>	LysM Receptor kinase
	TC98204	MtD03362	...	1.77	...	LRR receptor kinase
	TC95733	MtD04442	...	1.91	<i>MtSerk1</i>	BRI1-associated receptor kinase 1
	TC103374	MtC62903	...	1.95	<i>Mtpin1</i>	Auxin efflux carrier
	TC106906	MtD00718	...	1.84	...	GH3 auxin response protein
	TC101449	MtC61837	...	2.11	<i>Mtdvl1</i>	DVL protein
	TC102073	MtC90970	...	1.97	<i>Mtralf1</i>	Rapid alkalization factor

^a The MtGI8 column contains the TIGER8 gene index references and the MENS column contains the cluster references from the Medicago EST Navigation System.

^b Values are ratios of average expression in Nod factor (NF)-treated versus water-treated control (CF) samples; only ratios for NF-treated versus control samples greater than 1.6 with a *P* value < 0.05 are shown.

^c Markers = nodulation markers, TF = transcription factors, and Signal = signal perception and transduction.

those triggered by purified NF. Therefore, we performed a cross between the *sun-2* allele TR122 and *sickle* and isolated from the F2 progeny a double supernodulating line, *sun-2 sickle*. This double mutant showed an additive infection and nodulation phenotype (E.-P. Journet, *unpublished data*) similar to that described for a *sun-1 sickle* double mutant (Penmetsa et al. 2003). In order to test the response of this supernodulating line to NF and validate its use for the study of the NF-regulated transcriptome, we analyzed the expression in this mutant of the well-studied NF-responsive early nodulin gene *MtENOD11* (Charron et al. 2004). The analysis of a *MtENOD11*-promoter:β-glucuronidase (GUS) fusion showed that this supernodulating line has an increased response to NF (Fig. 1A). Indeed, not only is *MtENOD11* expression higher in the *sun-2 sickle* line than in a wild-type (WT) background but, in addition, the region of the root reacting to the NF treatment is broader (Fig. 1A). This result was further confirmed by a quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis. *MtENOD11* induction was approximately threefold higher in the *sun-2 sickle* line than in WT, both during early (6 h) and late (48 h) responses to NF (Fig. 1B). Therefore, we anticipated that the double supernodulating mutant *sun-2 sickle* should be a useful tool to study the NF-regulated transcriptome.

Identification of NF-responsive genes by microarray analysis.

Roots of young seedlings were treated either with 10^{-8} M NF or with water as a control and harvested after 6 and 48 h.

Fig 1. Double supernodulating mutant *sun-2 sickle* as a tool to study Nod factor (NF) responses. **A**, Histochemical β-glucuronidase (GUS) assay on wild-type (WT) A17 and *sun-2 sickle* supernodulating lines containing the GUS gene under the control of the *MtENOD11* promoter. The roots of the plantlets were treated with a 10^{-9} M NF solution for 6 h. **B**, Quantitative reverse-transcriptase polymerase chain reaction analysis of the expression of *MtENOD11* in roots of WT A17 and supernodulating *sun-2 sickle* line in response to a 10^{-8} M NF 6- and 48-h treatment. This figure represents one representative experiment from two biological repetitions that were qualitatively consistent. R stands for induction ratios between NF-treated roots versus roots before treatment (T0).

Corresponding cDNAs were then hybridized to microarrays containing 16,086 70-mer oligonucleotide probes representing more than 13,000 *M. truncatula* genes (Mt16kOLI1; discussed below). Using a cutoff of $P < 0.05$ and a minimum ratio of 1.6 for NF-treated versus control samples, the expression of 643 genes was found to be altered by NF treatment (265 upregulated and 378 downregulated). Two populations of early- and late-induced genes, showing a limited overlap (with only 12 and 21 genes induced or repressed, respectively, at both time points), could be distinguished.

To confirm the validity of our microarray data, we first checked the expression of marker genes shown by previous studies, other than transcriptomics, to be upregulated upon NF treatment. We detected an induction for three classical NF response markers, namely *MtANN1*, (de Carvalho Niebel et al. 1998), *RIP1* (Cook et al. 1995), and *MtENOD40* (Crespi et al. 1994; Fang and Hirsch 1998) (Table 1). Other markers such as *MtENOD11* or *MtNIN* are not represented on the Mt16KOLI1 microarrays and, therefore, could not be analyzed. In addition to nodulation markers (nodulins) known to be upregulated by NF, we also identified several nodulin and nodulin-like genes described as induced during symbiosis but that had not been shown to be NF-regulated before, such as *MtAnn2* (Manthey et al. 2004); *MtN1*-, *MtN3*-, *MtN6*-, and *MtN21*-like genes; *MtN24* (Gamas et al. 1996); *non symbiotic-leghemoglobin1*; and a gene highly homologous to a soybean symbiosome membrane nodulin gene (Table 1). It is interesting to note the presence in Table 1 of *MtLYK8* and *MtERN1*. *MtLYK8* is a member of the LysM receptor kinase gene family (Arrighi et al. 2006; Limpens et al. 2003) encoding potential NF receptors such as NFP or LYK3. *MtERN1* is an AP2/ERF transcription factor recently reported to be involved in NF signal transduction (Andrianakaja et al. 2007; Middleton et al. 2007). These results showed that the experimental conditions used in this study were well adapted to the identification of new NF-regulated genes.

We then compared our results with those published by Mitra and associates (2004), who used Affymetrix GeneChips representing approximately 10,000 *M. truncatula* genes to analyze responses 24 h after rhizobial inoculation or NF spot application in WT *M. truncatula*. Despite substantial differences in biological materials, experimental conditions, and expression profiling tools (some probes being present on only one type of microarray), we found 17 of the 40 (42%) NF-induced genes identified by Mitra and colleagues (Supplementary Table S2), as well as 248 additional genes. Mitra and colleagues identified only four repressed genes in their study, two of which were also identified by our approach. Consequently, nearly all of the clones we identified as repressed by NF (376 of 378) have never been described in an NF-regulation context before.

To validate our microarray data, we then performed qRT-PCR analyses with 10 genes coding for potential regulators, including proteins related to hormone signaling and transcription factors. We confirmed the up- or downregulation by NF of all of these genes in the *sun-2 sickle* background, which illustrates the reliability of our microarray data (Table 2). We then tested their expression pattern in WT *M. truncatula* and found that only half of them (5 of 10) were NF regulated as in the supernodulator *sun-2 sickle* line. We propose that this difference may be due primarily to detection limits for the WT resulting from the limited number of root cells responding or from the transient expression patterns of certain genes.

Identification of *MtRALFL1* and *MtDVL1*.

An intriguing observation was the upregulation by NF of two different genes encoding potential small regulatory peptides (Tables 1 and 2) never described so far in a symbiotic context. We hence decided to explore their possible involve-

ment during the symbiotic interaction between *M. truncatula* and *S. meliloti*.

MtRALFL1 (TC102073, MtC90970), encodes a 135-aa peptide that shows strong homologies to members of the RALF and RALF-like (RALFL) families of peptide hormones. This family comprises 39 members in *A. thaliana* and 40 in *M. truncatula* showing diverse expression patterns (Olsen et al. 2002; Silverstein et al. 2007). As for other described RALF peptides, *MtRALFL1* contains a predicted 27-aa-long N-terminal signal peptide, suggesting that this small peptide is secreted extracellularly (Pearce et al. 2001).

MtDVL1 (TC111142, MtC61837) is predicted to encode a small 53-aa peptide, without any clear signal peptide, homologous to the plant-specific family of potential regulatory peptides called DVL. In *A. thaliana*, *DVL* genes also belong to a large family of 21 members with partly overlapping functions (Wen et al. 2004). We identified only 10 *DVL* genes in the *M. truncatula* genome; however, this number is likely to increase as the genome sequence reaches completion.

Expression analysis of *MtRALFL1* and *MtDVL1*.

An expression analysis by qRT-PCR, using gene-specific primers, revealed the symbiotic expression pattern of *MtRALFL1* and *MtDVL1* (Table 2; Fig. 2), and sequencing of the PCR products confirmed that no other family members were amplified.

Both genes were found to be quite strongly upregulated in NF-treated roots of the supernodulator *sun-2 sickle* but not in WT plants (Table 2). *MtDVL1* but not *MtRALFL1* was found to be transiently upregulated in WT roots inoculated by *S. meliloti*, already 1 day post inoculation (dpi), which corresponds to the so called “preinfection stage”; that is to say, prior to penetration of the bacteria in root hairs (Fig. 2A and B). This modest (1.8-fold) but reproducible upregulation was confirmed using the *sun-2 sickle* line for which a much stronger (at least 6-fold) and longer induction was observed (Fig. 2D). In comparison, no significant upregulation of *MtRALFL1* expression was detected in *S. meliloti*-inoculated WT and *sun-2 sickle* roots (Fig. 2A and C). At later stages of the interaction (i.e., during nodule development), *MtRALFL1* showed a weak, transient, but reproducible upregulation in young developing nodules followed by a marked downregulation (3.3-fold) during subsequent stages of nodule development (Fig. 2E). A transient upregulation was reproducibly observed for *MtDVL1*. Indeed, corresponding transcripts were almost four times more abundant in young developing nodules than in roots (Fig. 2F). This difference then rapidly faded during nodule development, and mature (14 dpi) nodules showed the same *MtDVL1* expression level as noninoculated roots.

Thus, these data suggest that the expression of both *MtRALFL1* and *MtDVL1* are regulated by NF treatment or during early phases of nodule development or both.

Overexpression of *MtRALFL1* and *MtDVL1* affect rhizobial infection and nodulation.

In *A. thaliana*, plants carrying loss-of-function mutations or antisense or RNAi constructs for several *DVL* genes show no visible phenotype, probably because of functional redundancy within the large *DVL* gene family (Narita et al. 2004; Wen et al. 2004). Wu and colleagues (2007) used RNAi to silence *RALF* expression in *Nicotiana attenuata* and observed longer roots and abnormal root hair development. However, it is not clear from these data how specific their RNAi construct is and it cannot be excluded that more than one RALF gene is silenced in this case. To investigate the possible roles of *MtRALFL1* and *MtDVL1* during the legume–*Rhizobium* interaction, we performed overexpression studies, as with *AtDVL1-5* and *AtDVL16* (Rot4) in *A. thaliana* leaves (Narita et al. 2004; Wen et al. 2004). Using *Agrobacterium rhizogenes* transformation (Boisson-Dernier et al. 2005), we generated composite plants overexpressing *MtRALFL1* and *MtDVL1* in roots, under the control of the 35S promoter. We obtained a strong increase (on average, 38- and 23-fold, respectively) in *MtRALFL1*- and *MtDVL1*-overexpressing roots (Fig. 3A) compared with empty vector controls. No obvious phenotypic effect could be observed on root morphology or root growth when comparing overexpressing and empty vector control constructs (data not shown).

However, when transgenic roots were inoculated with *S. meliloti*, a clear reduction in the number of nodules formed was observed, with an average of 3- and 3.8-fold reduction for *MtRALFL1* and *MtDVL1* overexpressing roots, respectively, compared with empty vector controls (Fig. 3A). In addition, in *MtRALFL1* overexpressing roots, the few nodules formed had abnormal structures. These nodules remained very small, with poor rhizobial colonization (Fig. 4D), and never developed into mature nitrogen-fixing nodules. A fix[−] phenotype for these *MtRALFL1* overexpressing composite plants was revealed by their stunted development when grown in the absence of mineral nitrogen (data not shown). In contrast, nodule development and nitrogen fixation on inoculated roots overexpressing *MtDVL1* appeared normal when compared with control roots (Fig. 4E and F).

These observations led us to analyze rhizobial infection in the overexpressing transgenic roots. We discovered a strong increase in the number of abortive infections in roots overexpressing *MtRALFL1* and *MtDVL1* (10- and 7-fold, respectively) (Fig. 4C). In addition, the infection threads that generally aborted in

Table 2. Quantitative reverse-transcriptase polymerase chain reaction (q-RT-PCR) analysis of gene expression after Nod factor (NF) treatment^a

DFGI 8	MENS ID	SS SN F6/T0	SS SN F48/T0	A17 F6/T0	A17 F48/T0	Annotation
TC94919	MtC30198	202.35 **	53.96 **	29.27 *	13.66 *	β-Tubulin
TC96874	MtC50559	28.83 **	19.26 *	2.09 *	2.17 *	Putative BRI1-associated receptor kinase
TC96646	MtD05611	4.4 *	8.06 *	1.05	1.86 *	Myb (SHAQKYF class) transcription factor
TC103374	MtC62903	9.24 *	3.16 *	1.62 *	1.32	Auxin efflux carrier
TC102073	MtC90970	4.58 **	8.8 *	0.7	1.2	Rapid alkalization factor
TC111142	MtC61837	2.6 *	3.8 **	0.5	0.8	DVL protein
TC102505	MtC50581	3.95 *	4.21 *	0.78	1.13	HD-ZIP transcription factor
TC106906	MtD00718	2.98 **	2.85 *	0.79	1.12	GH3-auxin responsive gene
TC97459	MtD01753	1.75 **	1.72 *	0.46	1	EIN3
TC95807	MtC60974	0.38	0.33	0.1 *	0.23 *	Gibberellin-responsive gene

^a Confirmation by qRT-PCR analysis of upregulation after 6 and 48 h (F6 and F48, respectively) of NF treatment, in roots of the supernodulator *sun-2 sickle* line (SS SN) and wild-type *Medicago truncatula* (A17) for a selection of 10 potential regulator genes. Values are ratios of average expression levels for three biological replicates between NF-treated roots (F6 and F48) and roots prior to NF treatments (T0). The statistical significance of each expression ratio was calculated from the pairwise comparison between the expression levels in treated versus control samples, indicated by one or two asterisks corresponding to *P* < 0.05 or 0.01, respectively, according to the Student's *t* test. The MtGI8 column contains the TIGER8 gene index references and the MENS column contains the cluster references from the Medicago EST Navigation System.

the outer root cortex led to bulbous and sac-like arrested infection pockets (Fig. 4A through C).

DISCUSSION

sun-2 sickle as an improved tool to study NF-regulated responses.

The first objective of this study was to broaden our knowledge of the NF-regulated transcriptome in leguminous plants. The use of the double supernodulating mutant line, *sun-2 sickle*, has increased the sensitivity of this approach and provided a large collection of putative NF-regulated genes, including candidate signaling and regulatory genes, potentially involved in NF-signaling pathways.

In WT plants, AON takes place at early stages during NF signaling, before infection and possibly even before cortical

cell activation (Catford et al. 2003). In the supernodulating line *sun-2 sickle*, initial infection events are more frequent due to the *sickle* mutation (Penmetsa and Cook 1997) and AON is defective due to the *sun-2* mutation (Penmetsa et al. 2003; Schnabel et al. 2005). Overall, this leads to higher levels of infection and nodule organogenesis. Consequently, the transient accumulation of certain transcripts involved in NF-signaling is likely to be prolonged in these mutants. Such a phenomenon was already observed for *MtRIP1* in both *sun-1* and *sickle* by Penmetsa and colleagues (2003). In this respect, the late (48 h) time point for our NF transcriptome analysis in *sun-2 sickle* is of particular interest because it increases the likelihood of detecting low-abundance transcripts. Of course, it cannot be excluded at this stage that a certain number of the genes identified in our study are only NF regulated in either an ethylene perception or clavata-1-

Fig. 2. Quantitative reverse-transcriptase polymerase chain reaction expression analysis of *MtRALFL1* and *MtDVL1* during Nod factor (NF) response and nodulation in wild-type (WT) (A17) and in the supernodulating line *sun-2 sickle*. Expression analysis in response to NF of **A**, *MtRALFL1* and **B**, *MtDVL1* in entire WT A17 roots or isolated root hairs (RH) treated by water (H₂O) or NFs. **C**, **E**, and **G**, Analysis of *MtRALFL1* and **D**, **F**, and **H**, *MtDVL1* at different stages of the symbiotic interaction in whole root systems of **C** and **D**, WT (A17) and **E** and **F**, *sun-2 sickle* (SN) supernodulating lines 1, 2, or 3 days post inoculation (DPI) as well as **G** and **H**, in isolated nodules 4, 10, and 14 DPI. The values in all figures represent the average and standard deviation of three independent biological replicates. Numbers indicate the ratios between *MtRALFL1* or *MtDVL1* expression in treated roots or nodules versus control roots prior to inoculation or treatment (T0). Student's tests were used to estimate the significance of the observed differences between controls and treated samples. Asterisks are present when $P < 0.05$.

like deficient background, or both. However, these genes would not be devoid of interest because they may illustrate potential cross-talks between the NF and ethylene or AON pathways.

New insights into NF-regulated genes.

We are confident that the majority of the genes we list are truly NF regulated for several reasons. First, we have found in our list classical NF markers such as *MtENOD40*, *MtANN1*, *RIPI*, and also a recently described element of the NF-signaling pathway called *ERN1* (Andriankaja et al. 2007, Middleton et al. 2007). Second, a comparison with the only other large-scale study of NF-regulated genes (Mitra et al. 2004) shows that a significant proportion (approximately 40%) of the NF-regulated genes revealed by this previous study are present in our collection. Third, we have confirmed by qRT-PCR experiments the differential expression in *sun-2 sickle* for 10 selected genes, all coding for potential symbiotic regulators. This transcriptomic study has identified several other nodulin and nodulin-like genes known to be upregulated during nodulation but previously not described as being NF regulated. Importantly, among the list of 276 previously undescribed NF-repressed genes, we have identified over 50 potential regulators, including 15 transcription factors, nine kinases, four leucine-rich repeat receptor kinases, two phosphatases, two response regulator receptors, and several genes involved in auxin and gibberelin signaling (Supplementary Table S1). Although not the focus of this study, repressed regulators are likely to play equally important roles in NF signaling as upregulated regulators.

The potential role of *MtRALFL1* and *MtDVL1* in the *Rhizobium*-legume symbiosis.

Expression analyses of *MtRALFL1* and *MtDVL1* suggest that these genes are subject to early, transient, and localized NF-regulated expression. Overexpression experiments suggest, in addition, that both genes might have an impact on rhizobial infection during the initial progression of infection threads through the cortical root layers. Recent work questions the use of the 35S promoter for overexpression studies in mature nodules, where its expression is restricted to certain tissues (Auriac and Timmers 2007). In roots, however, the 35S promoter is strongly expressed in all root tissues (A. C. Timmers, *unpublished results*), allowing a clear conclusion about the impact of *MtRALFL1* and *MtDVL1* overexpression during rhizobial infection. For the majority of *M. truncatula* mutants described so far that affect rhizobial infection, such as *lin* (Kuppusamy et al. 2004), *ern* (*poodle*) (Middleton et al. 2007), or *nfp* and *dmi2* (revealed by RNAi) (Arrighi et al. 2006; Limpens et al. 2005), infection thread progression is altered either in the root epidermis or later in the inner cortex or even during bacterial release in the nodule infection zone (Veereshlingam et al. 2004). Thus, a predominant blockage of infection in the outer cortical layer, as observed for both *MtRALFL1* and *MtDVL1*, appears as an original phenotype and suggests a particular impact of these small peptides in this tissue.

In *Arabidopsis thaliana*, overexpression of *AtDVL1-5* and *AtDVL16* (*ROT4*) leads to pleiotropic phenotypes, including shortened stature, rounder rosette leaves, clustered inflorescences, shortened pedicles, and siliques with pronged tips (Narita et al. 2004; Wen et al. 2004). In addition, it has been shown that the reduced size of siliques and especially leaves is due to reduced cell proliferation. In the outer cortical cell layer, where most infection threads abort in roots overexpressing *MtDVL1*, rhizobia induce cell-cycle reactivation leading to the formation of cytoplasmic bridges, called preinfection-threads (PIT), through which infection threads then progress toward the inner cortex (Timmers et al. 1999; van Brussel et al. 1992). It would

be interesting to explore whether *MtDVL1* may be involved in or regulate cellular events leading to the differentiation of PIT.

Pierce and colleagues have shown that, when 10 μ M synthetic tomato RALF peptide is applied onto tomato and *A. thaliana* plantlets, a striking growth arrest is observed, blocking mer-

Fig. 3. Effects of *MtRALFL1* and *MtDVL1* overexpression on rhizobial infection and nodule formation. **A**, Quantitative reverse-transcriptase polymerase chain reaction analysis of the expression of *MtDVL1* and *MtRALFL1* in roots overexpressing these genes. Data represent pools of 14 independent transgenic lines. Numbers indicate the ratios between expression levels in roots transformed with overexpression constructs (35S) and empty vectors (control). **B**, Number of abortive infections in transgenic roots overexpressing *MtRALFL1* or *MtDVL1* (35S:*MtRALFL1*, 35S:*MtDVL1*) compared with empty vector transformed roots. Figures represent the average and standard deviation of infection thread counts of 17 transgenic roots revealed by a β -galactosidase assay. Bold numbers indicate the ratios between the number of abortive infections in roots transformed with overexpression constructs (35S:*MtRALFL1*, 35S:*MtDVL1*) and empty vector controls. **C**, Time course of nodule development (data is shown as nodules per root tip) between 0 and 20 days post inoculation (DPI) in roots transformed with overexpression constructs (35S:*MtRALFL1*, 35S:*MtDVL1*) and with an empty vector.

istem activity and root hair formation (Pearce et al. 2001). In addition, Wu and colleagues (2007) showed that silencing *RALF* in *N. alata* leads to the formation of longer roots. These results suggest a negative role for some RALF peptides on root growth and development. However, overexpression of *MtRALFL1* in transgenic *M. truncatula* roots did not lead to root growth arrest nor to any apparent effects on either root architecture or root and root-hair development. Furthermore, the marked symbiotic phenotype that we observed in the *MtRALFL1* overexpressing roots and nodules suggests that different *RALF* gene family members could play different roles during different developmental processes (i.e., rhizobial infection or root or nodule development). The presence of a pre-

dicted N-terminal signal peptide suggests that *MtRALFL1* encodes a secreted peptide. Therefore, it is conceivable that *MtRALFL1* is involved in cell-to-cell communication and, thus, in direct or indirect transduction of the NF signal. This hypothesis is particularly attractive in the light of a recent finding of Scheer and colleagues (2005), who identified two cell surface membrane proteins in tomato that could be part of a LeRALF receptor complex. Thus, we can speculate that overexpression of *MtRALFL1* might perturb this signal transduction between cells, leading to abnormal infection and possibly defects in nodule development.

Using a transcriptome approach based on the supernodulating line *sunn-2 sickle*, we have identified a large collection of

Fig. 4. Cytological analysis of the effects of *MtRALFL1* and *MtDVL1* overexpression on infection and nodule development. **A** through **C**, Infection thread (IT) morphology in root hairs and outer cortex of roots transformed with **A**, a *MtRALFL1* overexpression construct, **B**, empty vector control, and **C**, a *MtDVL1* overexpression construct. Arrows point to ITs in both **B**, a successful wild-type (WT) infection 3 days post inoculation (dpi) and roots 20 dpi showing arrested and abnormal ITs in transgenic roots overexpressing **A**, *MtRALFL1* or **C**, *MtDVL1*. **D** through **F**, Morphology of nodules 20 dpi developing on roots transformed with **D**, a *MtRALFL1* overexpression construct, **E**, empty vector control, and **F**, a *MtDVL1* overexpression construct. Scale bars represent 25 μ m (**A** through **C**) and 100 μ m (**D** through **F**).

NF-responsive genes in the model legume *M. truncatula*, representing a significant contribution to the description of NF responses in legumes. Our study also suggests a role for *MtDVL1* and *MtRALFL1* during nodulation and, in particular, during symbiotic rhizobial infection. Thus, in addition to more classical hormones such as ethylene, auxin, or cytokinin, these small potential regulatory peptides might also be required for early steps of the symbiotic *Rhizobium* spp.–legume interaction.

MATERIALS AND METHODS

Cultivation of plants, treatment, and harvesting of tissues.

The double mutant line *sun-2 sickle* was obtained by crossing the *sun-2* allele TR122 (Sagan et al. 1995) harboring the *pMtENOD11-GUS* construct to the sickle line (Penmetsa and Cook 1997). Both GUS-negative and GUS-positive plants were screened from the F2 progeny and selfed to produce homogeneous F3 populations. The double mutant character was genetically verified by test crosses to either parental single mutant.

Plant growth chamber conditions were as follows: temperature, 22°C; 75% hygrometry, light intensity, 200 $\mu\text{E m}^{-2} \text{s}^{-1}$; light and dark photoperiod, 16 and 8 h, respectively. WT *M. truncatula* A17 and supernodulating *sun-2 sickle* line plants were grown aeroponically during 11 days using a nitrogen-rich medium as described by Journet and associates (2001) with approximately 100 plants per caisson. Plants were then nitrogen starved for 4 days before being inoculated or NF treated. NF treatments were performed using a final concentration of 10^{-8} M WT (*S. meliloti*) NF solution or water for the control plants. Inoculations were performed with the *S. meliloti* GMI 6526 strain carrying a constitutive *hemA::lacZ* fusion as described by Ardourel and associates (1994) and grown at 30°C in tryptone yeast medium supplemented with 6 mM calcium chloride and tetracycline at 10 $\mu\text{g ml}^{-1}$, as described previously (El Yahyaoui et al. 2004).

Entire root systems were harvested 6 and 48 h after NF or water control treatments and at 0, 1, and 3 dpi by *S. meliloti*. Entire nodules were dissected and harvested 4, 10, or 14 dpi. All tissues were frozen and subsequently ground in liquid nitrogen.

Microarray hybridization.

Total RNA was isolated from NF- and water-treated roots using Trizol (Invitrogen, Cergy Pontoise Cedex, France), quantified using the Nanodrop technology (Wilmington, DE, U.S.A.) and qualitatively checked using a Bioanalyser (Agilent, Santa Clara, CA, U.S.A.). Total RNA (20 μg) was then used to synthesize Cy3- and Cy5-labeled cDNA targets as described by Küster and associates (2004). Mt16kOLI1 microarray hybridizations were performed using two independent biological replicates per experimental treatment. Hybridization of targets, image acquisition, and analysis of image data were performed as described previously (Hohnjec et al. 2005). Image processing data were analyzed using the EMMA 2.0 software (Dondrup et al. 2003). Lowess normalization was performed using a floor value of 20 and a Student's *t* test was applied to identify differentially expressed genes. Only those genes with a minimum expression ratio of 1.6 at $P < 0.05$ were considered that, in addition, were based on a minimum of 50% of nonflagged replicate spots.

The Mt16kOLI1 microarray definition file can be viewed on the European Bioinformatics Institute (EBI) website under accession number A-MEXP-85. In short, the microarray is composed of 16,086 70-mer oligonucleotide probes representing all TC of the TIGR *M. truncatula* gene index, release 5.0. Each probe is represented by two replicate spots per microarray. Complete transcriptome profile datasets can be viewed at the EBI website under accession numbers E-MEXP-943.

qRT-PCR.

For real-time qRT-PCR experiments, RNA was isolated using the SV total RNA extraction kit (Promega Corp., Madison, WI, U.S.A.), and also quantified using the Nanodrop technology and quality checked using a Bioanalyser (Agilent). Total RNA (3 μg) was reverse transcribed using the Superscript II enzyme from Invitrogen. Real-time PCR was then performed on a Light cycler (Roche, Mannheim, Germany) using Syber-Green and the following primers: MtDVLqF (GGAGCAAAGGGCTAGGCTAT), MtDVLqR (TGAACGGTGGACACTGCTAC), MtRALFqF (TCCAACCCAAAAACAACACA), and MtRALFqR (GGGAA GTGCAGATTGGAAGA), amplifying a 200-bp fragment between position 136 and 336 of expressed sequence tag (EST) cluster MtC61837 for *MtDVL1* and a 105-bp fragment between position 10 and 115 of EST cluster MtC90970 for *MtRALFL1*. Specificity of amplification was then tested by sequencing. The values were normalized with an internal control, *EF1- α* : (MtGI8: TC106470 and MENS: MtD00010.1_GC), using primers EF1 α F: CTTTGCTTGGTGCTGTTTAGATGG and EF1 α R: ATTCAAAGGCGGCTGCATA verified to be expressed at a similar level in the tested conditions. Three biological replicates were used for each sample and the data shown correspond to the mean value and standard deviation of these three repeats.

Overexpression experiments.

Overexpression of *MtDVL1* and *MtRALFL1* was performed by inserting a 145- and 407-bp fragment, respectively, into pPEX, a pGreen-based vector containing DsRed as a reporter gene (Combiér et al. 2006) using the *Xho1-Not1* restriction sites after amplification with the following primers: MtDVL5'*Xho* (TCAGTCCGCTCGAGATGAAGATGGGTGC TATTGGAGG), MtDVL3'*Not* (AAGGAAAAAGCGGCC GCTTACTCATGCCAACATAGAAGC), MtRALF5'*Xho* (TC AGTCCGCTCGAGATGTCCCAACTCAGATTCACCT), and MtRALF3'*Not* (AAGGAAAAAGCGGCCGCTTAAGTGT TATTTCTTGCGCAC).

Both constructs and empty control vectors were introduced into *Agrobacterium rhizogenes* Arqual strain and root transformations of *M. truncatula* were then performed as described by Boisson-Dernier and associates (2005). Transgenic plants were transferred into growth pouches 3 weeks after transformation and supplemented with caisson growth medium as described by Journet and associates (2001). Composite plants were inoculated with *S. meliloti*, 1 week after transfer.

Observation and counting of infection threads.

The presence of infecting *hemA::lacZ* bacteria was revealed by performing a β -galactosidase activity test using 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Biosynth AG, Staad, Switzerland) as described previously (Boivin et al. 1990). Infection threads were counted at 20 dpi, and those not leading to nodule development at such a late stage were counted as abortive infection threads.

Histochemical GUS staining.

GUS stainings were performed as described by Journet and associates (2001).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

European Bioinformatics Institute website:
www.ebi.ac.uk/microarrayexpress
 The Institute for Genomic Research website: www.tigr.org
 Medicago EST Navigation System (MENS) database:
medicago.toulouse.inra.fr/Mt/EST