ROOT DETERMINED NODULATION1 Is Required for M. truncatula CLE12, But Not CLE13, Peptide Signaling through the SUNN Receptor Kinase[OPEN]

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The combinatorial interaction of a receptor kinase and a modified CLE peptide is involved in several developmental processes in plants, including autoregulation of nodulation (AON), which allows legumes to limit the number of root nodules formed based on available nitrogen and previous rhizobial colonization. Evidence supports the modification of CLE peptides by enzymes of the hydroxyproline O-arabinosyltransferase (HPAT/RDN) family. Here, we show by grafting and genetic analysis in Medicago truncatula that, in the AON pathway, RDN1, functioning in the root, acts upstream of the receptor kinase SUNN, functioning in the shoot. As expected for a glycosyltransferase, we found that RDN1 and RDN2 proteins are localized to the Golgi, as was shown previously for AtHPAT1. Using composite plants with transgenic hairy roots, we show that RDN1 and RDN2 orthologs from dicots as well as a related RDN gene from rice (Oryza sativa) can rescue the phenotype of rdn1-2 when expressed constitutively, but the less related MrRDN3 cannot. The timing of the induction of MtCLE12 and MtCLE13 peptide genes (negative regulators of AON) in nodulating roots is not altered by the mutation of RDN1 or SUNN, although expression levels are higher. Plants with transgenic roots constitutively expressing MtCLE12 require both RDN1 and SUNN to prevent nodule formation, while plants constitutively expressing MtCLE13 require only SUNN, suggesting that the two CLEs have different requirements for function. Combined with previous work, these data support a model in which RDN1 arabinosylates MtCLE12, and this modification is necessary for the transport and/or reception of the AON signal by the SUNN kinase.

When available nitrogen compounds in the soil are limiting to plant growth, legumes can undergo nodulation to take advantage of atmospheric nitrogen. Nodulation involves species of rhizobia bacteria entering the roots of legumes and inducing the formation of structures called nodules to house the bacteria, fixing atmospheric nitrogen for the plant in exchange for photosynthetically derived carbon skeletons from the plant. The symbiosis involves extensive signaling within the plant as well as between the two partners in order to establish and maintain the relationship. As reviewed by Oldroyd and Dixon (2014), the establishment of the symbiosis requires both positive and negative regulation by the plant. While the benefit to the plant of nitrogen from the atmosphere is high, the cost of the symbiosis to the plant (estimated at 12 g of carbon per 1 g of nitrogen fixed [Crawford et al., 2000]) makes the symbiosis only cost-effective under nitrogen-limiting conditions. Nitrogen-limiting conditions are detected systemically by the plant through C-terminally encoded peptides sent from the root to the shoot (Imin et al., 2013) to interact with receptors such as CRA2 (Huaault et al., 2014) and initiate nitrogen-acquiring responses such as nodulation and the up-regulation of nitrate transporters while acting locally to control lateral root initiation (Taleski et al., 2016).

The initiation of nodule development occurs when flavonoids released from the root trigger the release of Nod Factor from the rhizobia. Reception of the species-specific Nod Factor by a set of receptors leads to engulfment of the bacteria and the onset of cell division in the inner cortex. The bacteria pass through the cell layers of the root inside an infection thread, while inner cortical cells divide and form a meristem for the future nodule. When the infection thread reaches these dividing cells, rhizobia are released into membrane-bound compartments called symbiosomes, where they fix nitrogen for the plant (Oldroyd and Dixon, 2014).
Autoregulation of nodulation (AON) allows the plant to limit the number of nodules formed based on available nitrogen and previous rhizobial colonization. Grafting experiments with mutant plants demonstrated that nodule regulation in the roots was controlled from the shoot (Postma et al., 1988), and subsequent research has shown that factors in both the root and the shoot, as well as the transmission of signals between these parts of the plant, contribute to the ability to regulate nodule number (for review, see Shabala et al., 2016).

In *Medicago truncatula*, a model legume for studying indeterminate nodulation, in which the meristem continues to grow throughout the lifespan of the nodule, gene products demonstrated by mutation or overexpression to regulate nodule number include SICKLE, an EIN2 ortholog regulating from the root (Penneta et al., 2008), SUNN, a CLV1-like receptor kinase expressed throughout the plant that acts from the shoot to control AON (Penneta et al., 2003; Schnabel et al., 2005), CRA2, a Leu-rich repeat receptor regulating systemically from the shoot and locally within the root (Huault et al., 2014), and peptides MtCLE12p and MtCLE13p, affecting AON through expression in the root (Mortier et al., 2010). These peptides are likely transported to the shoot (Okamoto et al., 2013) and have been shown to require SUNN for their effects (Mortier et al., 2012). Also demonstrated to be involved in AON by mutation is *RDN1*, expressed throughout the plant vasculature but shown by grafting to modulate AON from the root (Schnabel et al., 2011). The AON signal in *M. truncatula* is generated in the root upon the perception of rhizobia, and genetic evidence points to *MtCLE12*, *MtCLE13*, *RDN1*, and the cytokinin receptor CRE1 influencing that signal (Mortier et al., 2010; Plet et al., 2011; Schnabel et al., 2011). The signal (presumably the transport of the CLE peptides [Okamoto et al., 2013]) is perceived by the SUNN receptor kinase in the shoot. In coordination with *MtCLV2* and *MtCRN* (Crook et al., 2016), further nodulation is restricted based on the development of the nodules and the nitrogen needs of the plant. Legumes send a second signal to the root to stop further nodule development, and this signal involves the transport of both auxin (van Noorden et al., 2006) and cytokinin (Gonzales et al., 2005; Sasaki et al., 2014). In the determinate nodulator *Lotus japonicus*, where nodules cease growing during the life of the plant, perception of the downstream signal in the roots requires the Kelch repeat F-box protein TML (Takahara et al., 2013), but in plants that nodulate indeterminately, this has not been shown.

When first identified as a conserved protein of unknown function, *RDN1* was the founding member of a small gene family conserved in all green plants, although the effect of mutation in *M. truncatula* was limited to the loss of AON and shorter roots (Schnabel et al., 2011). The proteins in the gene family were identified subsequently from Arabidopsis (*Arabidopsis thaliana*) as hydroxyproline O-arabinosyltransferases (HPATs), and the data are consistent with the enzymes adding the first of several arabinose molecules to some CLE peptides and extensins (Ogawa-Ohnishi et al., 2013).

Based on its role in AON and the information from Arabidopsis, we hypothesized that *RDN1* arabinosylates one or both of the *MtCLE* peptides involved in nodulation signaling. Based on work in *L. japonicus*, we suspect that this modification is necessary for transport and/or reception of the signal, presumably by the SUNN kinase. In the *L. japonicus* system, RS-CLE1 and RS-CLE2 require the SUNN ortholog HAR1 to suppress nodulation (Okamoto et al., 2009). When ectopically added to the cotyledons of *L. japonicus*, RS-CLEs lacking arabinosylation are unable to inhibit nodulation compared with RS-CLEs carrying arabinose residues (Okamoto et al., 2013), but the details of how the RS-CLEs are modified and transported to the shoot have not yet been determined.

To support our hypothesis that *RDN1* is the arabinosyltransferase modifying root-derived CLE signals, we determined the order of action of *RDN1* and SUNN by reciprocal grafting of the mutants, inverted-Y grafts, and genetic analysis of double mutants. We found that *RDN1* acts before the receptor kinase SUNN. We found that *RDN1* and *RDN2* proteins are localized to the Golgi. In addition, we demonstrate that *RDN1* and *RDN2* orthologs from dicots as well as a related *RDN* gene from rice (*Oryza sativa*) can rescue the phenotype of *rdn1-2* when expressed constitutively in roots of composite transgenic plants, but the less related *MdRDN3* cannot. The timing of the induction of the regulating *MtCLE12* and *MtCLE13* peptide genes in nodulating roots is not altered by mutation of *RDN1* or SUNN, although expression levels are higher. Plants with transgenic roots constitutively expressing *MtCLE12* require both *RDN1* and SUNN to prevent nodule formation, while plants constitutively expressing *MtCLE13* require only SUNN, suggesting that the two CLEs have different modification requirements for function. Combining our data with previous work, we propose a model in which *RDN1* arabinosylates *MtCLE12*, and this modification is necessary for the transport and/or reception of the AON signal by the SUNN kinase.

**RESULTS**

**RDN1 Is Involved in Sending the Initial AON Signal from the Root**

The determination that *RDN1* acts in AON from the root was based on reciprocal grafting (Schnabel et al., 2011), which cannot determine if *RDN1* is involved in sending the AON signal to the shoot or receiving the AON signal from the shoot. To resolve this uncertainty, we created plants with inverted-Y grafts (Kassaw and Frugoli, 2012), in which a root of a different genotype is grafted onto a plant to test the systemic effect of inoculation of one root on the second root of a different genotype (Fig. 1A). The genotype of the second root has no effect on the nodule number on the first root, as the roots are inoculated sequentially; the first root is already developing nodules when the second root is inoculated. As described previously (Kassaw and Frugoli, 2012), when two wild-type roots shared a wild-type shoot and...
the second root was inoculated 4 d after the first, the second root showed a decrease in nodule number due to a long-distance suppression signal from the first root, which was already nodulating (Fig. 1B, group 1). Likewise, when two rdn1-2 mutant roots shared an rdn1-2 mutant shoot and were inoculated 4 d apart, both roots hypernodulated and no decrease in nodule number was observed in the second root (Fig. 1B, group 4). These two examples are the inverted-Y graft controls. Interestingly, with a wild-type shoot, when the first root inoculated was wild type and the second root inoculated was rdn1-2, normal regulation was observed (Fig. 1B, group 2). Specifically, nodule number on the rdn1-2 second root was statistically the same as on the wild-type second root in Figure 1B, group 1. This suggests that the rdn1-2 mutant root can perceive and respond to the normal nodule suppression signal initiated by the root sent through the wild-type shoot. When the first root inoculated and the shoot came from an rdn1-2 mutant and the second root inoculated came from a wild-type plant (Fig. 1B, group 3), the wild-type second root had nodules statistically intermediate in number between that from wild-type first roots naive to a signal (black bars in Fig. 1B, groups 1 and 2) and second roots receiving a wild-type signal (gray bars in Fig. 1B, groups 1 and 2). Since rdn1-2 has been shown to affect nodule number exclusively from the root (Schnabel et al., 2011), this is consistent with rdn1-2 having a defect in the suppression signal going to the shoot from the first inoculated root. Together, these data support rdn1 mutants

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Grafting experiments demonstrate that RDN1 sends the AON signal rather than receives it. A, Schematic of the construction of a split-root plant (two roots of different genotypes) by insertion of a root cut from genotype B into a notch in a root of genotype A. B, Nodule number on inverted-Y grafted plants when the wild type (wt) acts as the main stock contributing the shoot and one of the roots (1 and 2) or rdn1 acts as the main stock contributing the shoot and one of the roots (3 and 4). The second root is inoculated 4 d after the first root. Error bars indicate SE; letters indicate significant differences using the Tukey-Kramer minimum significance test ($P < 0.05$). $n = 3$ to 16 plants per graft.
Figure 2. Grafting experiments and genetics demonstrate that RDN1 and SUNN act in the same pathway. A, Reciprocal grafting of wild-type (wt), sunn-4, and rdn1-2 shoots and roots. Plants were grown on perlite, and data were collected 21 d post rhizobial inoculation (Kassaw and Frugoli, 2012). Data are mean nodule numbers for n = 11 to 18 successful grafts per combination. Error bars indicate se; letters indicate significant differences using the Tukey-Kramer minimum significance test (P, 0.05). B, Mean nodule number of wild-type and single and double mutant plants grown in the same aeroponic chamber, n = 3 to 6 plants per genotype. Error bars indicate se; letters indicate significant differences using the Tukey-Kramer minimum significance test (P, 0.05). C, Representative photograph of plants from the experiment in B, three per genotype.
being defective in sending the suppression signal to the shoot, not receiving the suppression signal from the shoot.

RDN1 and SUNN Function in the Same Genetic Pathway

Since SUNN and RDN1 are postulated to function in the same pathway while exerting their effects from different parts of the plant, we employed shoot-to-root reciprocal grafting to investigate the order of action. We created reciprocal grafts between the null alleles sunn-4 and rdn1-2, using each genotype as shoot or root. As expected, given that RDN1 acts in the root and SUNN acts in the shoot, rdn1-2 shoot and sunn-4 root reciprocal grafts showed wild-type nodulation (Fig. 2A); the mutations have no effect when placed in the opposite orientation from their known locations of action. Self-grafts of sunn-4 produced a significantly higher number of nodules than self-grafts of rdn1-2 (Fig. 2A), consistent with the respective mutant phenotypes of nongrafted plants (Schnabel et al., 2010, 2011). However, reciprocal grafts between sunn-4 shoots and rdn1-2 roots have the same nodule number as sunn-4 self-grafts, not rdn1-2 self-grafts (Fig. 2A), suggesting that the effect of a SUNN mutation on nodulation from the shoot demonstrated...
by Penmetsa et al. (2003) overrides the effect of an RDN1 mutation from the root demonstrated by Schnabel et al. (2011). This result would be expected if RDN1 is involved in generating the signal that is sent to SUNN.

We reasoned that if RDN1 modifies CLE peptides that bind to the SUNN receptor, as suggested by Ogawa-Ohnishi et al. (2013), genetic analysis also should support SUNN and RDN1 operating in a common genetic pathway. Plants containing mutations in both SUNN and RDN1 were generated for pathway analysis. These include two null alleles of RDN1 (Schnabel et al., 2011), the original SUNN allele carrying an amino acid change (sum1-1; Schnabel et al., 2005), and a null allele of SUNN (sum4-4) with a stronger phenotype (Schnabel et al., 2010). The sum1-1;rdn1-1 double mutant plants have the same nodule number phenotype as the sum4-4 null mutant plants and significantly higher nodule numbers than both the sum1-1 and rdn1-1 parental single mutants (Fig. 2B). Root length in the sum1-1;rdn1-1 plants also phenocopied that of sum4-4 mutant plants (Fig. 2C). Therefore, both phenotypes show an additive effect of the null rdn1-1 allele on the weak sum1-1 allele. The sum4-4; rdn1-2 double mutant plants carrying null mutations in both genes showed enhanced nodule number compared with the rdn1-2 parent but not compared with the parental single mutant sum4-4; the absence of both genes did not create additive effects on nodule number when compared with the single parental sum4-4 phenotype, consistent with RDN1 and SUNN functioning in the same genetic pathway.

RDN1 Is Associated with Secretory Pathway Components

The RDN1 protein has an N-terminal signal peptide predicting involvement in the secretory pathway in plants (Schnabel et al., 2011). Arabidopsis HPAT1 was identified in proteomics analyses in plasma membrane fractions (Marmagne et al., 2007; Mitra et al., 2009) and vacuolar fractions (Carter et al., 2004; Jaquinod et al., 2007), while others found localization in the Golgi using a GFP-tagged version of the protein in protoplasts of their respective plant systems (Ogawa-Ohnishi et al., 2013; Xu et al., 2015). Since the biological activity of the GFP-tagged proteins was not reported in these investigations, we created a plasmid expressing RDN1-GFP under the control of the 35S cauliflower mosaic virus (CaMV) promoter (see “Materials and Methods”). The RDN1-GFP fusion protein was expressed in rdn1-1 and rdn1-2 mutant roots via Agrobacterium rhizogenes transformation to confirm its functionality by phenotypic rescue. The construct complemented the rdn1-2 mutant allele when compared with the control roots transformed with an empty vector (Fig. 3A). The construct was then observed in both wild-type and rdn1-2 hairy roots. RDN1-GFP localized in tiny moving organelles in the cytoplasm of M. truncatula root hair cells (Fig. 3B). The punctate pattern of GFP fluorescence in Figure 3B streamed through the cytoskeleton in a Brownian fashion (Supplemental Fig. S1D).

To confirm that the observed organelles were Golgi, the RDN1-GFP fusion protein was expressed transiently in tobacco (Nicotiana tabacum) leaves coinfiltred with various organelle-specific markers developed in Arabidopsis (Nelson et al., 2007). While not colocalized with plasma membrane markers, RDN1-GFP was observed at the edge of the internal side of the plasma membrane (Fig. 3, C–E), consistent with endosomal trafficking. In addition, RDN1-GFP did not colocalize with an mCherry-tagged endoplasmic reticulum marker (Fig. 3, F–H). However, the RDN1-GFP fusion protein clearly colocalized with the Golgi mCherry-tagged protein marker (Fig. 3, I–K). The same punctate localization observed in Figure 3B was observed for gene family members RDN2 in M. truncatula cells and the poplar (Populus trichocarpa) sequence ortholog of RDN1 (Supplemental Fig. S1, A–C). Taken together, these results confirm that native RDN1 and RDN2 enzymes are localized in the Golgi/endosomal system.

RDN Enzymes from Other Species Can Substitute for RDN1 When Constitutively Expressed

The RDN enzymes share sequence homology between species that suggests orthology, but there is not a one-to-one correspondence of genes within the family (Schnabel et al., 2011). To determine if the enzymes could substitute for each other within or across species if expressed appropriately, we transformed the M. truncatula rdn1-2 mutant roots with wild-type copies of genes with the highest amino acid similarity to RDN1 from M. truncatula, poplar, rice, and Arabidopsis (Supplemental Fig. S2A). When MlRDN2, PtRDN, OsRDN, and AtHPAT3
were expressed under the control of the CaMV 35S promoter, in each case the nodule regulatory defect of rdn1-2 mutants was corrected to a level consistent with rescue by MtRDN1 (Fig. 4), with statistically lower nodule numbers than the rdn1-2 mutant transformed with an empty vector (P < 0.05, Tukey-Kramer test). In contrast, one RDN family member in M. truncatula, MtRDN3, did not rescue the rdn1-2 mutation when expressed under the control of the 35S promoter (Supplemental Fig. S2B), suggesting a functional divergence of RDN gene paralogs in M. truncatula.

The CaMV 35S promoter is a strong constitutive promoter, chosen to give high levels of gene expression in dicot plants; we reasoned that the similarity in nodule regulation responses between legume and nonlegume RDNs could be due to the high expression levels obtained when homologous sequences were expressed under the control of this promoter. Therefore, we expressed all the genes mentioned above except MtRDN3 under the control of the native MtRDN1 promoter used previously (Schnabel et al., 2011). We found that MtRDN1 restored AON under the control of its own native promoter (Supplemental Fig. S2C). However, only OsRDN could restore AON to the same level as MtRDN1 when expressed this way. Nevertheless, MtRDN2, PtRDN, and AtHPAT3, while not statistically different from the control, showed intermediate nodule number regulation compared with the AON-defective mutant (B group in Supplemental Fig. S2C), suggesting that these enzymes have some overlapping function but cannot completely restore AON expressed from the MtRDN1 native promoter.

Figure 5. The expression of MtCLE12 and MtCLE13 is induced to a higher level in rdn1-2 and sunn-4 mutants. Wild-type (wt) A17, sunn-4, and rdn1-2 seedlings were inoculated with Sinorhizobium medicae and harvested at 0, 3, 10, and 15 d after inoculation. MtCLE12 (A) and MtCLE13 (B) expression in roots was measured by quantitative real-time PCR. The level of expression of each gene was calculated relative to the expression of the reference gene (see “Materials and Methods”). Data are means of three technical replicates each of two biological replicates, and error bars indicate maximum SE.
Expression of MtCLE12 and MtCLE13 in rdn1-2 and sunn-4 Mutants Is Increased But Retains the Wild-Type Temporal Pattern of Induction

Both the rdn1-2 and sunn-4 mutants have increased nodule primordia, leading us to expect that more nodule meristems would lead to increased expression of MtCLE12 and MtCLE13 compared with wild-type plants. Indeed, a nodulation time course showed almost no expression of MtCLE12 and MtCLE13 in rdn1-2 and sunn-4 mutants before inoculation with rhizobia, just as in wild-type plants (Fig. 5). The expression results for wild-type plants agree with those reported by Mortier et al. (2010). At 3 d post inoculation, the expression of both genes increased in wild-type and mutant plants. At 10 d post inoculation, the expression of both genes increased further in wild-type and mutant plants, although the mutant plants increased more relative to the control gene. At 15 d, a point at which nodules mature and begin to fix nitrogen in this system (Kassaw et al., 2015), the expression of both genes remained within the range of error of the 10 d level in all plants.

Constitutive Expression of MtCLE12 and MtCLE13 Yields Different Phenotypes in an rdn1-2 Background

Initial evidence that SUNN could be the receptor for the MtCLE12 and MtCLE13 peptides comes from the observation that constitutive expression of MtCLE13 in transgenic hairy roots of a sunn-4 mutant had no effect on sunn-4 nodule number while nearly eliminating nodulation in wild-type plants (Mortier et al., 2012). We reasoned that if SUNN was also required for perception of the MtCLE12 peptide, constitutive expression of MtCLE12 in M. truncatula hairy roots should reduce nodule number in wild-type plants and have no effect in transgenic hairy roots of sunn-4 plants, an effect we were able to confirm (Fig. 6, A and B). Furthermore, if the MtCLE12 and MtCLE13 peptides require arabinosylation and RDN1 encodes the enzyme that arabinosylates the CLE peptides, we should see the same lack of effect on nodulation when the peptides are constitutively expressed in rdn1-2 transgenic hairy roots. Constitutive expression of MtCLE12 had no effect in rdn1-2 roots, but to our surprise, constitutive expression of MtCLE13 in transgenic hairy roots reduced the nodule number to wild-type levels in rdn1-2 mutants in three independent experiments.

DISCUSSION

Based on its role in AON and the information from Arabidopsis, we hypothesized that RDN1 modifies one or both of the MtCLE peptides involved in nodulation

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**Figure 6.** Constitutive expression of MtCLE12 and MtCLE13 in rdn1-2 and sunn-4 mutants has different effects. Nodule number is shown for perlite-grown plants with transgenic hairy roots expressing MtCLE12 or MtCLE13 under the control of the CaMV 35S promoter inoculated with S. medicae and nodules counted 21 d later. Three independent experiments are indicated with dark gray, light gray, and white bars; B has only two replicates, as it is a repeat of a published experiment (see text).

A, Wild type A-17; n = 4 to 8 plants per construct per experiment. B, sunn-4; n = 4 to 9 plants per construct per experiment. C, rdn1-2; n = 4 to 9 plants per construct per experiment. Error bars indicate SE, and asterisks indicate significance (P < 0.001, Student’s t test).
signaling and that this modification is necessary for the transport and/or reception of the signal by the SUNN kinase. Our evidence presented here supports the model in Figure 7, explained below, in which the MtCLE12 peptide produced in nodule meristems is modified by the RDN1 enzyme in the Golgi before transport to the shoot, while the MtCLE13 peptides are not modified by RDN1. However, both MtCLE12 and MtCLE13 signal through the SUNN kinase.

In support of the model in Figure 7, we previously showed that RDN1 affects nodulation from the root (Schnabel et al., 2011), and here, we show that the RDN1 gene acts before SUNN by inverted-Y grafting experiments (Fig. 1) and in the same pathway by reciprocal grafting and mutational analysis (Fig. 2). In the model (Fig. 7A1), green indicates the inner cortical cells across from the xylem pole at the root hair curling stage that will become the nodule meristem (Geurts and Bisseling, 2002) and express MtCLE12 and MtCLE13 (Mortier et al., 2010). Figure 7A2 shows a closeup of these green cells, where CLE peptides are expressed as prepropeptides and modification by the addition of arabinose chains is predicted to occur in the Golgi (Tabata et al., 2014). We show that RDN1 is localized to the Golgi, consistent with RDN1 being an enzyme that modifies CLE peptides (Fig. 3), and vesicles containing RDN1 appear adjacent to the plasma membrane, similar to secretory vesicles of the endomembrane system (Fig. 3, E and K). Our data do not shed light on how MtCLE12 and MtCLE13 leave the cell. However, CLV3 is localized to the extracellular space to bind CLV1 (Rojo et al., 2002), so this is likely a common mechanism for CLE action. Experiments in M. truncatula exposing CLE36 to extracellular fluids suggest that the final modification of the prepropeptide to a short 12- to 13-amino acid peptide may occur by a protease in the apoplast (Djordjevic et al., 2011). Once reaching the xylem, MtCLE12 and MtCLE13 are transported to the shoot, based on experiments in an L. japonicus/soybean (Glycine max) hybrid system (Okamoto et al., 2013).

Upon reaching the shoot (Fig. 7B3), the MtCLE12 and MtCLE13 peptides bind to the SUNN receptor kinase. The ability to stop nodulation in a SUNN-dependent manner when constitutively expressing MtCLE12 and MtCLE13 in roots provides genetic support for SUNN as the MtCLE12 and MtCLE13 receptor (Fig. 6, A and B; Mortier et al., 2012). SUNN interacts with MtCLV2 and MtCRN, and mutations in these genes in L. japonicus and M. truncatula result in hypernodulation (Krusell et al., 2012).
from expression level differences in the 35S constructs. the two genes have similar native levels of expression for arabinosylation to aid binding. However, given that the amount of peptide produced through constitutive reduced a
CLEs to their receptors is limited to one peptide and one effeciency of arabinosylation on the binding afunction of the SUNN kinase. Since the demonstrated ef-
does not require arabinosylation by RDN1 to signal
that either the MtCLE13 peptide does not require ara-
biosylation.

CONCLUSION

Genetic evidence supports a model in which the RDN1 enzyme modifies the MtCLE12 peptide involved in nodulation signaling and this modification is necessary for transport and/or reception of the signal by the SUNN kinase. The MtCLE12 peptide produced in nodule meristems is modified by the RDN1 enzyme in the Golgi before transport to the shoot, while the MtCLE13 peptide is either not modified by RDN1 or does not require modification for function. However, both MtCLE12 and MtCLE13 signal through the SUNN kinase. Together, these data indicate that the requirement for modifications of CLE peptides for function, as well as the determination of which enzymes modify which peptides, is not a simple relationship. Given the number of CLE peptides and receptor kinases in plants, as well as a family of RDN arabinosyltransferases in all green plants, much work remains to understand the many signal transduction pathways that use these common components.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of Medicago truncatula 'Jemalong A17', the AON-defective mutants rdn1-1, rdn1-2 (Schnabel et al., 2011), sunn-1, and sunn-4 (Schnabel et al., 2005), as well as the double mutants sunn-1;rdn1-1 and sunn-4;rdn1-2 were acid scarified and imbibed as described (Schnabel et al., 2010). Seed was then ver-
nalized in the dark at 4°C for 2 d on Harrison modified Farhaeus medium (Huo et al., 2006), covered with two half-round Whatman filter papers (GE Healthcare), followed by germination in the dark at room temperature for 1 d before being used in the experiments described. M. truncatula plants used for phe-
notypic analysis and comparison of the double mutants were grown in an aeroponic chamber in nodulation medium (Pemneta and Cook, 1997). The shoot-to-root reciprocal grafting experiments were performed as described (Kassaw and Frugoli, 2012).

Agrobacterium rhizogenes-Mediated Plant Transformation

Seedlings were transformed as described previously (Limpens et al., 2004). The hypocotyls of 5-d-old seedlings were cut and transformed by lightly scraping on the surface of Luria-Bertani plates densely grown in A. rhizogenes strain Arual (Quandt et al., 1993) containing the appropriate binary vector and antibiotic selections (at 30°C for 48 h). After 5 d of cocultivation with the A. rhizogenes in the growth chamber at 23°C (16 h of light and 8 h of dark), the seedlings were transferred to nutrient-rich hairy root emergence medium (Limpens et al., 2004) containing 300 µg ml⁻¹ cefotaxime (Phytotechnology Laboratories) sandwiched between two half-round Whatman filter papers grown under the same growth conditions. Five days later, the top filter papers were removed from the plates and the seedlings were allowed to grow for an additional 5 d on the same emergence medium placed vertically in the same growth chamber. For nodulation experiments, nontransgenic roots were trimmed off and the entire plant was transferred to Perlite as described (Kassaw and Frugoli, 2012). The plants were watered for 5 d with a 100-fold dilution of water-soluble 20:10:20 Peat-Lite Special fertilizer (Scotts). Fertilization was then withdrawn, and the plants were hydrated with water alone for an additional 5 d in order to induce
Plasmid Construction for Subcellular Localization Studies

The RDN1 coding sequence (MaT7p5g08520) was amplified from M. truncatula ‘Jemalong A17’ cDNA using the primers listed in Supplemental Table S1. PCR products were gel extracted and purified using the Zymoclean gel DNA recovery kit (Zymo Research). The Gateway BP and LR recombination reactions were used to clone the RDN1 coding sequence without the stop codon per the manufacturer’s instructions. The RDN1 PCR product was cloned into pDONR221 vector to generate a Gateway entry clone utilizing kanamycin (50 μg mL⁻¹) as the selective agent, and each of the resulting entry clones was characterized by restriction mapping and sequence analysis. A single correct clone was used for the work.

To generate the expression clone under the control of the CaMV 35S promotor, the LR reaction was performed using the entry clone and the pK7FWG2 destination vector (Karimi et al., 2002) suitable for C-terminal eGFP fusion to the RDN1 protein. In addition, the destination vector was reconstituted to use the native RDN1 promotor by removing and replacing the CaMV 35S promoter with the native promotor by restriction digestion, resulting in the expression of RDN1 under the control of its own promotor. The functionality of the constructs was tested by rescue of the rdn1 nodulation phenotype using A. rhizogenes-mediated hairy root transformation as described (Schnabel et al., 2011). The organelle-specific markers Golgi-mCherry, ER-mCherry, and PM-mCherry were obtained from the Arabidopsis Biological Resource Center (Nelson et al., 2007). These expression constructs were used for both A. rhizogenes-mediated hairy root transformation and transient tobacco (Nicotiana tabacum) leaf infiltration.

Plasmids for Cross-Species Rescue

The Gateway cloning system was used to clone all rescue constructs. cDNAs prepared from M. truncatula, poplar (Populus trichocarpa), and rice (Oryza sativa) were used to prepare recombination vectors to amplify RDN1, RDN2, RDN3, PRD1, and OsRDN1 genes. The primers used are listed in Supplemental Table S1. PCR products were cloned as above into the pK7FWG2 destination (expression) vector (Karimi et al., 2002), creating a C-terminal eGFP fusion to all the proteins. In addition, the pK7FWG2-R destination vector containing the DsRED fluorescent reporter gene was altered to use the MIRRD1 promoter to drive expression by removing the CaMV 35S promotor through restriction digestion and replacement as above. Vectors containing the proper constructs were transformed into A. rhizogenes strain Arqua1 (Quardt et al., 1993) for hairy root transformation.

Transient Protein Expression in Tobacco

The binary vectors described in plasmid constructs were introduced into Agrobacterium tumefaciens (EHA105; Hood et al., 1993) by electroporation, and transformed cells were selected on Luria-Bertani plates supplemented with kanamycin (50 μg mL⁻¹) for organelle-specific markers and spectinomycin (50 μg mL⁻¹) for the Gateway plasmids carrying MIRRD1 at 30°C. An individual colony from each construct was inoculated into 3 mL of liquid TY medium and grown at 30°C in a shaker at 250 rpm for 48 h. Cells were harvested by centrifugation and resuspended in 1 mL of infiltration medium (10 mM MgCl₂ and 100 μM acetylsyringone). The cells were further washed twice with 1 mL of infiltration solution by spinning for 2 min at 5,500g. The resuspended 1 mL of A. tumefaciens solution was incubated at least 2 h at room temperature in the infiltration solution without shaking. The bacterial solution was then diluted to OD₆₀₀ = 0.4 and injected into young fully expanded leaves of 4- to 5-week-old tobacco plants grown under greenhouse conditions using a 1-mL syringe without a needle by gentle pressure through the small incision made on the lower epidermal surface. Transformed plants were incubated on a light rack with regular watering under 16 h of light and 8 h of dark. Three to 7 d after infiltration, leaves were examined for localization experiments using an Axiovert 200 M fluorescence microscope with Apotome (Carl Zeiss) and a laser scanning confocal imaging system.

Confocal Microscopy Imaging and Analysis

Transformed leaves and transgenic roots were imaged with an inverted Zeiss LSM 510 laser scanning microscope and plan-apochromat 63×/1.4 oil differential interference contrast objectives. For imaging coexpression of the GFP constructs and mCherry-tagged organelle-specific markers, excitation lines of an argon ion laser of 488 nm for GFP and 543 nm for mCherry were used with a 505/530-nm band-pass filter for GFP and 560-nm long-pass filter for mCherry in the single-track facility of the microscope. Postacquisition image processing was performed with the LSM 5 Image Browser (Carl Zeiss).

Quantitative Real-Time PCR

Total RNA was isolated from nodulating roots with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. Each RNA sample was digested with RNase-free DNase (Promega) treatment for 40 min to remove genomic DNA contamination. The iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize cDNA from 1 μg of RNA in a 20-μL reaction. The cDNAs were then diluted to 40 μL. All experiments were performed with iQ SYBR Green Super Mix and the iCycler iQ5 multicolor detection system (Bio-Rad). Each reaction was performed in triplicate, and results were averaged for a single biological replicate. The total reaction volume was 12.5 μL (10 μL of master mix including 0.175 μL of each primer [0.35 μM final concentration] and 2.5 μL of cDNA). Cycle threshold values were obtained with the accompanying software, and expression was determined relative to the SECRET AGENT gene (Schnabel et al., 2005). Primers are shown in Supplemental Table S1. These primers are unique in the M. truncatula genome based on National Center for Biotechnology Information Primer-BLAST analysis.

CLE Overexpression

Transgenic roots expressing MiCLE12 and MiCLE13 under the control of the CaMV 35S promotor were constructed and analyzed as described above (“Agrobacterium rhizogenes-Mediated Plant Transformation”). Plants were transformed with the plasmids used by Mortier et al. (2012), a gift of Sofie Goormachtig.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. In planta localization of RDN-GFP proteins.

Supplemental Figure S2. Cross-species rescue is dependent on both the gene and the promotor.

Supplemental Table S1. List of primers used in the study.

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