The Autoregulation Gene *SUNN* Mediates Changes in Root Organ Formation in Response to Nitrogen through Alteration of Shoot-to-Root Auxin Transport[1][W][OA]

Jian Jin[2], Michelle Watt, and Ulrike Mathesius*

Australian Research Council Centre of Excellence for Integrative Legume Research, Division of Plant Science, Research School of Biology, Australian National University, Canberra, Australian Capital Territory 0200, Australia (J.J., U.M.); and Commonwealth Scientific and Industrial Research Organization Plant Industry, Black Mountain Laboratories, Canberra, Australian Capital Territory 2601, Australia (M.W.)

We tested whether a gene regulating nodule number in *Medicago truncatula*, *Super Numeric Nodules* (*SUNN*), is involved in root architecture responses to carbon (C) and nitrogen (N) and whether this is mediated by changes in shoot-to-root auxin transport. Nodules and lateral roots are root organs that are under the control of nutrient supply, but how their architecture is regulated in response to nutrients is unclear. We treated wild-type and *sunn-1* seedlings with four combinations of low or increased N (as nitrate) and C (as CO_2) and determined responses in C/N partitioning, plant growth, root and nodule density, and changes in auxin transport. In both genotypes, nodule density was negatively correlated with tissue N concentration, while only the wild type showed significant correlations between N concentration and lateral root density. Shoot-to-root auxin transport was negatively correlated with shoot N concentration in the wild type but not in the *sunn-1* mutant. In addition, the ability of rhizobia to alter auxin transport depended on N and C treatment as well as the *SUNN* gene. Nodule and lateral root densities were negatively correlated with auxin transport in the wild type but not in the *sunn-1* mutant. Our results suggest that *SUNN* is required for the modulation of shoot-to-root auxin transport in response to altered N tissue concentrations in the absence of rhizobia and that this controls lateral root density in response to N. The control of nodule density in response to N is more likely to occur locally in the root.

Nitrogen (N) is one of the limiting nutrients for plant growth, and plants show large phenotypic plasticity in the response to N availability in the environment. N is available as nitrate or ammonium from the soil, and many legumes additionally gain N from a symbiosis with N-fixing bacteria called rhizobia. Rhizobia initiate the development of root nodules in specific legume hosts. They invade the nodules and convert atmospheric N into ammonia, which is exported to the plant as amino acids. In return, the plant provides rhizobia with a carbon (C) source because N fixation has high energy demands (White et al., 2007).

Little is known about the genetic regulation of the developmental plasticity responses to N. Some of the first identified genes regulating N phenotypes are a class of genes regulating nodule number by a systemic mechanism called autoregulation (Reid et al., 2011b). Mutation of these autoregulation genes causes supernodulation, mostly even in the presence of high N concentrations, and were thus first termed *nitrate tolerant symbiotic* (*nts*) mutants in soybean (*Glycine max*; Carroll et al., 1985a, 1985b).

In legumes, the number and density of nodules on a root system are strictly regulated to balance the need for N with the supply of C as an energy source for N fixation. A Leu-rich repeat receptor-like kinase is responsible for the systemic regulation of nodule number and density. This Leu-rich repeat receptor-like kinase, also named Nodulation Autoregulation Receptor Kinase (NARK), has been cloned from different legumes, including the crops soybean (Searle et al., 2003) and pea (*Pisum sativum*; Krusell et al., 2002) and the model legumes *Lotus japonicus* (Krusell et al., 2002; Nishimura et al., 2002) and *Medicago truncatula* (Schnabel et al., 2005). Autoregulation mutants, in which NARK is dysfunctional, are unable to down-regulate nodule number, and their roots are supernodulated

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[2] Present address: Key Laboratory of Black Soil Ecology, Northeast Institute of Geography and Agro-ecology, Chinese Academy of Sciences, Haping Road, Harbin 150081, China.

* Corresponding author; e-mail ulrike.mathesius@anu.edu.au.

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with high densities of nodules along the whole root system. Grafting experiments have shown that NARK acts in the shoot to regulate nodule density by long-distance signaling (Delves et al., 1986). Split-root experiments demonstrated that infection of the root by rhizobia induces a signal that moves to the shoot and activates NARK, and subsequently, a signal is sent back from the shoot to the root to inhibit further nodule development, at least temporarily (Kosslak and Bohlool, 1984). Interestingly, autoregulation mutants also show the *nts* phenotype (Carroll et al., 1985b; Sagan et al., 1995); that is, they still nodulate in the presence of nitrate concentrations that inhibit nodulation in the wild type, although various mutants differ in their degree of nitrate tolerance. Again, grafting studies showed that the *nts* phenotype is exerted by the shoot in soybean and *M. truncatula* (Day et al., 1989; Sagan et al., 1995; Jeudy et al., 2010). However, there is also a local effect of N supply on nodulation in *M. truncatula* that affects nodule size and color (Jeudy et al., 2010). Therefore, the inhibitory action of nitrate on nodulation is expected to interact with the autoregulation signal, perceived through NARK, to downregulate nodule density. It is likely that NARK directly or indirectly perceives the C/N ratio in the shoot and then sends a long-distance signal to the root to adjust nodule density according to N demand and C supply. Several autoregulation mutants also show nodulation-independent phenotypes, for example, an increased density of lateral roots and a short root system (Wopereis et al., 2000), suggesting that these phenotypes are also under the control of a long-distance signaling system. So far, the long-distance signals that regulate nodule or lateral root density have not been identified, but auxin is one candidate for a shoot-to-root signal regulating nodule and lateral root development.

Auxin is mainly synthesized in the shoot and transported to the root via phloem transport and by active polar cell-to-cell transport (Friml, 2003). Auxin is a crucial regulator of lateral root (Fukaki et al., 2007) and nodule (Mathesius, 2008) development. Lateral root initiation is dependent on auxin transport from the root tip to the lateral root initiation zone (Reed et al., 1998; Casimiro et al., 2001), whereas auxin transport from the shoot to the root is required for lateral root elongation (Bhalerao et al., 2002).

The regulation of lateral root initiation and elongation by nitrate is likely to be mediated by auxin signaling. In general, local patches of high nitrate availability trigger lateral root elongation, whereas high systemic levels of N in the plant cause an inhibition of lateral root emergence (Zhang and Forde, 1998; Zhang at al., 1999; Forde, 2002).

Studies focusing on the local stimulation of lateral root elongation in response to nitrate showed that the auxin-resistant Arabidopsis (*Arabidopsis thaliana*) mutant *arx4* is resistant to nitrate-induced lateral root elongation (Zhang et al., 1999), although this was not confirmed in a different study (Linkohr et al., 2002). The demonstration that the nitrate transporter (NRT1.1) also transports auxin and thus directly links nitrate sensing with lateral root elongation strongly links local auxin transport into an elongating lateral root with nitrate sensing (Krouk et al., 2010). In addition, application of a synthetic auxin transport inhibitor above the site of localized nitrate supply in Arabidopsis prevented the outgrowth of lateral roots, suggesting that auxin transport is involved in the lateral root response to nitrate (Guo et al., 2005).

Studies examining the inhibition of lateral root emergence in response to high (generally more than 10 mM) systemic treatment with nitrate showed that high-nitrate treatment causes changes in auxin concentration and response in the root. For example, microarray and other gene expression experiments provide evidence for the regulation of many auxin response and auxin transport genes in nitrate-treated Arabidopsis plants (Gutiérrez et al., 2007; Gifford et al., 2008). The repression of lateral root initiation in Arabidopsis seedlings grown under high-C and low-N conditions was linked to high auxin response in the hypocotyl and low response in the root and could be rescued by auxin addition to the roots (Malamy and Ryan, 2001). Shifting roots from high- to low-nitrate medium increased root auxin content in pineapple (*Ananas comosus*; Tamaki and Mercier, 2007) and in Arabidopsis, where this increase was accompanied by increased lateral root emergence (Walch-Liu et al., 2006). In maize (*Zea mays*), nitrate treatment led to the inhibition of root growth, and this was correlated with reduced auxin concentration in the root, particularly close to the root tip (Tian et al., 2008). Bao et al. (2007) showed reduced expression of the synthetic auxin-responsive promoter:GUS fusion, DR5:GUS, in response to high nitrate, suggesting that the auxin response might be affected by nitrate.

To our knowledge, no genes mediating systemic effects of N or C on lateral root development have been identified. However, autoregulated nodulation genes have been suggested as candidates (Walch-Liu et al., 2005).

As for lateral root formation, there is evidence that auxin transport control is required for nodule development. Local auxin transport regulation is required for nodule initiation, and young nodule primordia show cell-specific auxin responses (Mathesius et al., 1998; Pacios-Bras et al., 2003; van Noorden et al., 2007). In *M. truncatula* roots in which the cell cycle regulator *CDC16* was silenced, reduced auxin response was linked to lower numbers of lateral roots but increased numbers of nodules, supporting the hypothesis that these two processes require different auxin concentrations (Kuppusamy et al., 2009). In soybean, the auxin content of the roots was increased by inoculation with rhizobia but reduced in the presence of nitrate in the wild type, whereas auxin concentrations in an autoregulation mutant did not respond to nitrate or inoculation (Caba et al., 2000). There is also evidence that auxin acts as a long-distance signal from the shoot to

the root. The *M. truncatula* autoregulation mutant *super numeric nodules-1* (*sunn-1*) shows increased shoot-to-root auxin transport compared with the wild type. After inoculation of *M. truncatula* wild-type roots, shoot-to-root auxin transport is inhibited at the same time as the autoregulation starts. This down-regulation of auxin transport was not found in the *sunn-1* mutant, suggesting that shoot-to-root auxin transport inhibition could be part of the long-distance autoregulation control (van Noorden et al., 2006).

These studies suggest that auxin could be a long-distance signal that regulates both lateral root and nodule development in response to N and possibly C supply. To examine this question, we utilized the *sunn-1* mutant because of its known phenotype of altered shoot-to-root auxin transport and defects in regulating root organ numbers. We grew *M. truncatula* wild-type and *sunn-1* mutant plants under high and low nitrate and CO_2 regimes to test (1) whether the *sunn-1* mutant shows altered N-response phenotypes, (2) whether the C or N supply alters shoot-to-root auxin transport, (3) whether this altered auxin transport is correlated with altered root growth and lateral root and nodule formation, and (4) whether *SUNN* mediates the changes in root architecture to altered C or N supply via the regulation of shoot-to-root auxin transport.

RESULTS

SUNN Affects Growth Responses to C and N

Our first aim was to determine whether the *SUNN* gene affects the plant responses to externally applied C and N. Therefore, we grew wild-type and *sunn-1* mutant seedlings under four different C and N conditions: approximately ambient C (ambient C; 370 μL L^{-1} CO_2 in the atmosphere); high C (800 μL L^{-1} CO_2 in the atmosphere); minus N (no external N source); and plus N (2.5 mM nitrate in the growth medium). This nitrate concentration was chosen because it was sufficient to inhibit nodulation (data not shown) and N fixation (Moreau et al., 2008) in the wild type to the same extent as higher (5 or 10 mM) N concentrations and at the same time increased internal N concentrations significantly (see below). As increasing nitrate concentrations delay nodulation in *M. truncatula* (Moreau et al., 2008), we kept nitrate concentrations to a minimum to avoid the asynchronous behavior of plants with different nitrate treatments. Half of the plants were inoculated with rhizobia and the other half were mock inoculated 5 d after germination. Root and shoot material was collected at day 20 after germination, at which time pink nodules had formed in both genotypes in the absence of N. We determined the fresh weight of shoots and roots as well as N concentrations (Fig. 1), C concentrations, and C and N contents (Supplemental Fig. S1).

In general, *sunn-1* seedlings were smaller than wild-type seedlings, especially the root systems (Fig. 1A). Whereas the wild type showed significantly increased shoot weight in response to N and high C plus N, *sunn-1* mutants showed only moderate increases in shoot weight in response to N and only significantly responded to high C plus N. Root weight increased significantly in response to high C and high C plus N in both genotypes. In both genotypes, inoculation of roots with rhizobia caused a significant reduction in root and shoot weight in high-C-plus-N-treated plants but not in the other treatments.

While application of C and N did not significantly affect C concentrations in any of the treatments or genotypes (Supplemental Fig. S1A), it changed N concentrations significantly (Fig. 1B). In both genotypes, 2.5 mM nitrate significantly increased N concentrations in shoots and roots. Treatment with high C plus N reduced N concentrations significantly compared with ambient C plus N in wild-type shoots and roots but only in *sunn-1* mutant shoots. The *sunn-1* seedlings generally showed higher N concentrations in shoots and roots compared with the wild type (Fig. 1B).

In summary, these results suggest that, even though *sunn-1* seedlings had higher N tissue concentrations compared with the wild type and N concentrations increased in response to nitrate treatment, *sunn-1* seedlings generally responded less strongly to N addition with changes in biomass.

SUNN Affects Root Architecture Responses to C and N

Our next question was whether the *SUNN* gene mediates changes in root architecture in response to C and N. We determined nodule density, lateral root density, and taproot and lateral root length in wild-type and *sunn-1* seedlings grown under the C and N treatments described above (Fig. 2). Under our growth conditions, *sunn-1* roots developed significantly more nodules and higher nodule density than wild-type roots. In both genotypes, nodule densities were significantly reduced in response to N addition and significantly increased in response to high C. High C did not rescue the low-nodulation phenotype of the plus-N treatment (Fig. 2A).

Lateral root density at ambient-C and minus-N conditions was similar between genotypes. Nitrate addition significantly reduced the lateral root density in the wild type, but this was not the case in *sunn-1* mutants (Fig. 2B). High C significantly increased lateral root density in uninoculated wild-type plants, while this effect was only significant in minus-N treatments in *sunn-1* mutants.

Lateral root length was generally shorter in *sunn-1* mutants than in the wild type (Fig. 2C). While N addition significantly increased lateral root length in the wild type, this response only occurred with a combination of high C plus N in *sunn-1* mutants and was much less pronounced. Inoculation significantly reduced lateral root length in all treatments in the wild type but only in the high-C treatments in *sunn-1* mutants.

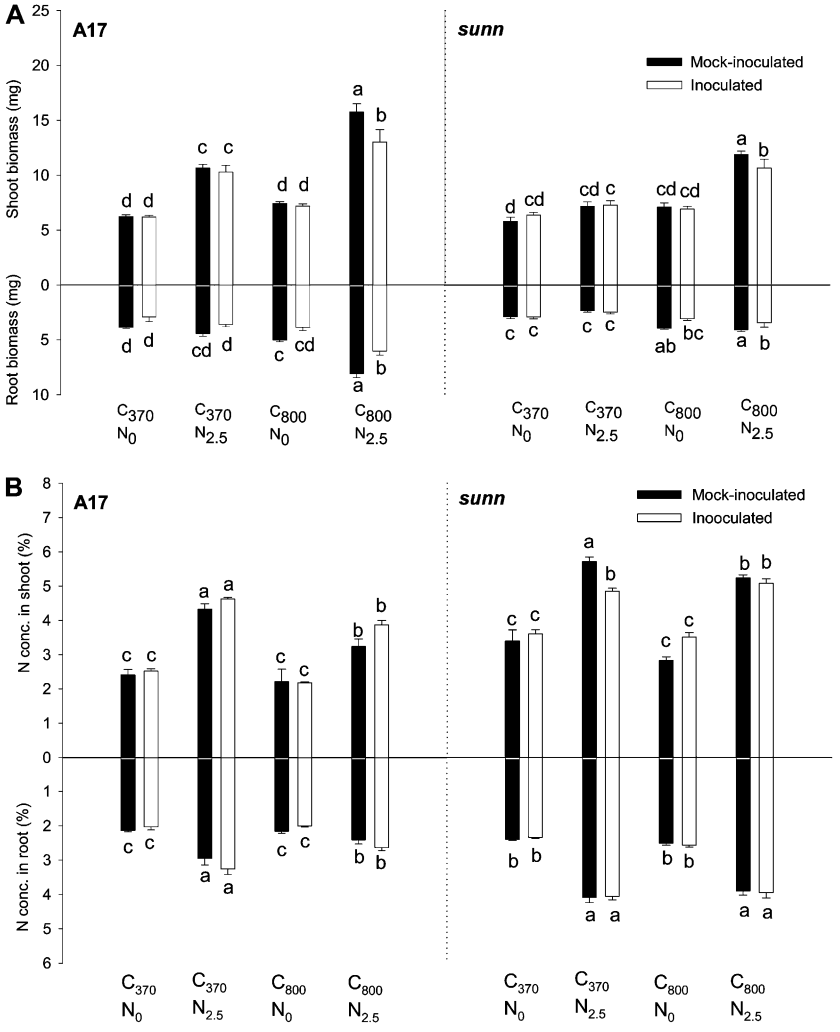

Figure 1. Biomass and N concentrations of cv Jemalong A17 and *sunn-1* seedlings. A, Shoot and root biomass (in mg dry weight). B, N concentrations of A17 (wild type) and *sunn-1* shoot and roots determined by mass spectrometry. Data are expressed as percentage dry weight. Data were obtained from 20-d-old A17 and *sunn-1* seedlings grown in ambient C (370 μL L^{-1} CO_2 [C_{370}]), high C (800 μL L^{-1} CO_2 [C_{800}]), minus N (0 mM nitrate [N_0]), and/or plus N (2.5 mM nitrate [$N_{2.5}$]). Inoculation with *S. meliloti* was performed on 5-d-old seedlings. Data are results from four samples of six to eight seedlings each ± SE. Treatments labeled with different letters differ significantly within one genotype ($P < 0.05$; two-way ANOVA).

Taproot length was not significantly affected by C or N in the wild type but was reduced by ambient C plus N in *sunn-1* mutants (Supplemental Fig. S2).

To test whether root organ density and root length are related to the availability of C or N in the shoot or root, we correlated these root architecture phenotypes to C and N tissue concentrations. We show here a correlation with shoot [N], but in all cases very similar results were found for root [N], and in both genotypes shoot [N] and root [N] were strongly positively correlated (Supplemental Fig. S3A). In both wild-type and *sunn-1* seedlings, nodule density was strongly negatively correlated with shoot [N] ($P < 0.001$; Fig. 3A). There was no significant correlation of nodule density with shoot (or root) [C] (Supplemental Fig. S3B). Lateral root density was strongly negatively correlated with shoot [N] in the wild type ($P < 0.01$) but not in *sunn-1* mutants (Fig. 3B). There was no significant correlation between lateral root density and shoot or root [C] (Supplemental Fig. S3C). In both genotypes, lateral root length showed no significant correlation with either shoot or root [N] or [C] (data not shown). Taproot length showed a slight but significant ($P < 0.05$) negative correlation with shoot [N] in both genotypes (Fig. 3C) but no response to shoot [C] (data not shown).

In short, these results suggest that the *SUNN* gene is required for changes in lateral root density in response to [N], while nodule density and taproot length are affected by shoot [N] similarly in both genotypes.

SUNN Is Required for Changes in Shoot-to-Root Auxin Transport in Response to [N]

One of our hypotheses was that root organ density was determined by the amount of shoot-to-root auxin transport, which we previously found to be regulated by the *SUNN-1* gene under conditions of N limitation and ambient CO_2 (van Noorden et al., 2006). We now wanted to determine whether this shoot-to-root auxin transport mediated changes in root architecture in response to C and N. Measurements of shoot-to-root auxin transport in 10-d-old mock-inoculated seedlings showed (1) that *sunn-1* mutants had significantly

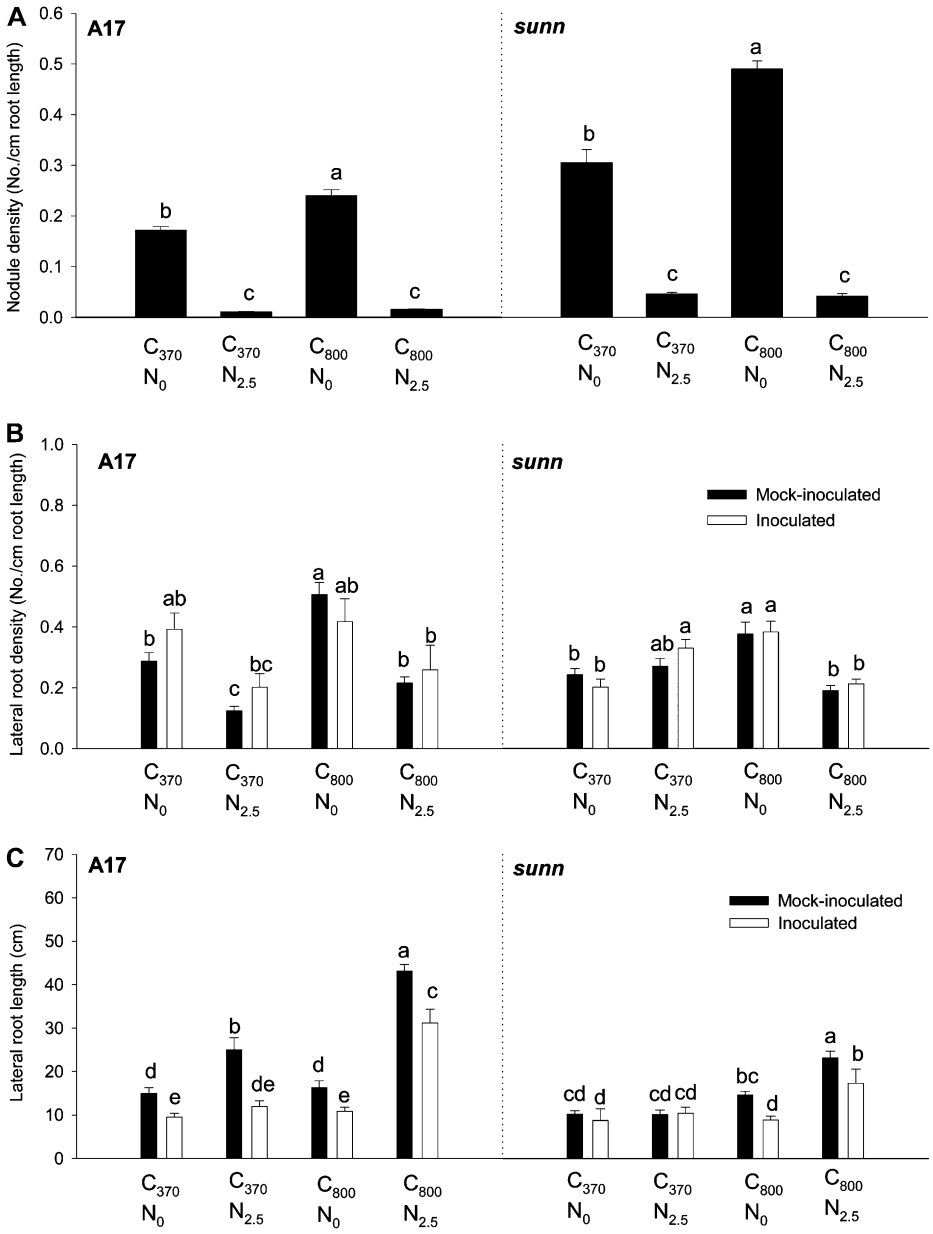

Figure 2. Root architecture phenotypes of A17 and *sunn-1* seedlings. Nodule density (A), first-order lateral root density (B), and lateral root length (total of all lateral roots per plant; C) of 20-d-old cv Jemalong A17 and *sunn-1* seedlings grown in ambient C (370 μL L^{-1} CO_2 [C_{370}]), high C (800 μL L^{-1} CO_2 [C_{800}]), minus N (0 mM nitrate [N_0]), and/or plus N (2.5 mM nitrate [$N_{2.5}$]) are shown. Inoculation with *S. meliloti* was performed on 5-d-old seedlings. Data are results from four samples of six to eight seedlings each ± SE. Treatments labeled with different letters differ significantly within one genotype ($P < 0.05$; two-way ANOVA).

higher auxin transport than the wild type, as reported previously (van Noorden et al., 2006), (2) that treatment with 2.5 mM nitrate increased auxin transport in the wild type but not in *sunn-1* mutants, and (3) that supply of high C reduced auxin transport, although this could be rescued by N addition in the wild type (Fig. 4A). To further examine the relationship between C and N supply and shoot-to-root auxin transport, we correlated total auxin transport with shoot and root [N] and [C]. We found positive and statistically significant ($P < 0.001$) correlation between shoot (and root; data not shown) [N] with total auxin transport in the wild type but not in *sunn-1* mutants (Fig. 4B). There was no significant correlation of auxin transport with shoot (or root) [C] (Fig. 4C). This result suggests that the *SUNN* gene is required for the modulation of shoot-to-root auxin transport in response to N tissue concentrations.

SUNN Couples Shoot-to-Root Auxin Transport Regulation to Root Organ Density

To find out whether long-distance auxin transport could determine the density of root organs formed, we correlated auxin transport with the density of emerged lateral roots and nodules. For this comparison, we examined 6- and 10-d-old seedlings, which had been inoculated 5 d after germination (i.e. measurements were made 24 and 120 h after [mock] inoculation). The 24-h time point was chosen because at this time,

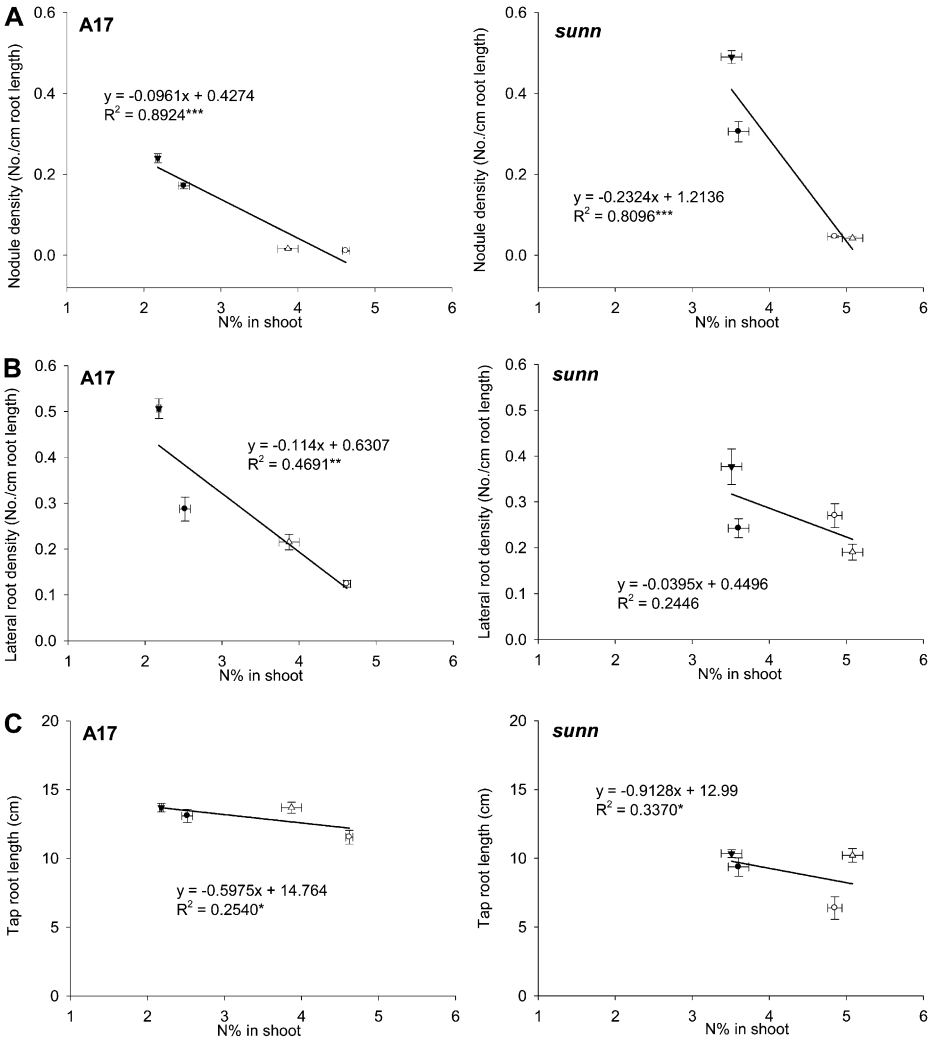

Figure 3. Correlation of shoot N concentrations with root architecture phenotypes. A, Correlation between nodule density and shoot N concentration. B, Correlation between lateral root density (of mock-inoculated roots) and shoot N concentration. C, Correlation between taproot length (of mock-inoculated roots) and shoot N concentration. N concentrations are expressed as percentage N of dry weight. All data are from 20-d-old cv Jemalong A17 and *sunn-1* seedlings grown in ambient C (370 μL L^{-1} CO_2 [C_{370}]), high C (800 μL L^{-1} CO_2 [C_{800}]), minus N (0 mM nitrate [N_0]), and/or plus N (2.5 mM nitrate [$N_{2.5}$]). Inoculation with *S. meliloti* was performed on 5-d-old seedlings. Data are results from four samples of six to eight seedlings each ± SE. Significant correlations are marked with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ [calculated with linear regression]).

autoregulation (i.e. temporary inhibition of nodule formation) was determined to start in *M. truncatula*, and this was associated with a drop in shoot-to-root auxin transport in the wild type but not in *sunn-1* mutants (van Noorden et al., 2006). After 120 h, nodule formation resumed in the wild-type and lateral roots started to emerge.

Comparison of auxin transport in mock-inoculated compared with inoculated seedlings showed that in the wild type, rhizobia caused a significant decrease in auxin transport after 24 h in ambient-C and no-N conditions, as reported previously (data not shown; van Noorden et al., 2006). After 120 h, auxin transport increased after inoculation in the no-N treatments (which formed nodules) but significantly decreased in the plus-N treatments (which formed very few or no nodules; compare with Fig. 2; Supplemental Fig. S4). In *sunn-1* seedlings, inoculation with rhizobia did not significantly alter auxin transport in any of the treatments (Supplemental Fig. S4). Correlation of total auxin transported in 10-d-old inoculated seedlings with nodule density showed a slight but significant ($P < 0.05$) negative correlation in wild-type seedlings but not in *sunn-1* seedlings (Fig. 5A). When correlating the difference in auxin transport between inoculated and mock-inoculated seedlings with the density of nodules formed, we found a significant ($P < 0.001$) and positive correlation (Fig. 5B) in the wild type (i.e. a significant increase in auxin transport 120 h after inoculation was correlated with higher nodule density). Again, this correlation was absent in *sunn-1* seedlings. These data indicate that *SUNN* is required for the alteration of auxin transport by rhizobia, as found by van Noorden et al. (2006), and that this change in auxin transport in inoculated roots could be responsible for controlling the density of nodules formed.

Correlation of lateral root density with auxin transport in mock-inoculated seedlings showed a significant ($P < 0.01$) negative correlation in the wild type but not in *sunn-1* mutants (Fig. 5C). Lateral root length was significantly positively correlated with auxin transport in the wild type but negatively correlated in *sunn-1* mutants (Fig. 5D). These results suggest that total shoot-to-root auxin transport influences lateral

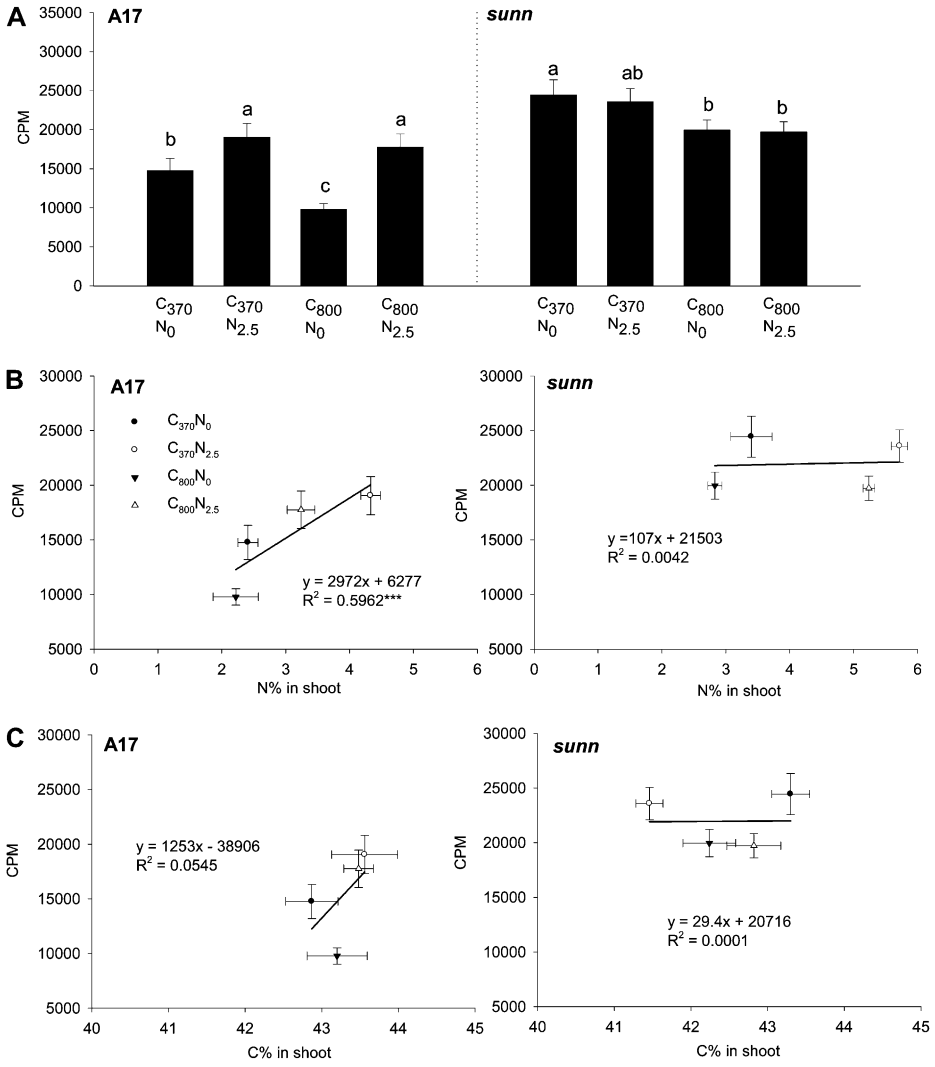

Figure 4. Shoot-to-root auxin transport phenotypes in response to C and N treatments. A, Total transported [^{3}H]IAA (in cpm) in 10-d-old, mock-inoculated cv Jemalong A17 and *sunn-1* seedlings grown in ambient C (370 μL L^{-1} CO_2 [C_{370}]), high C (800 μL L^{-1} CO_2 [C_{800}]), minus N (0 mM nitrate [N_0]), and/or plus N (2.5 mM nitrate [$N_{2.5}$]). Radiolabeled auxin was applied between the cotyledons at the shoot apex and allowed to transport into the root. After 3 h, eight 5-mm segments, starting just below the cotyledons, were excised into scintillation fluid. Segment 1 was not taken into account for the analysis because of diffusion from the site of application. The results show the sum of the total amount of radiolabeled auxin transported into segments 2 to 8 as means ± SE (n = 16–20). Treatments labeled with different letters differ significantly within one genotype ($P < 0.05$; one-way ANOVA). B, Correlation of total shoot-to-root auxin transport with N concentration in the shoot. C, Correlation of total shoot-to-root auxin transport with C concentration in the shoot. Significant correlations are marked with asterisks (*** $P < 0.001$ [calculated with unbalanced linear regression]).

root density and length and that this depends on the function of *SUNN*.

DISCUSSION

In this study, we wanted to test whether a nodule autoregulation gene, *SUNN*, plays a role in other aspects of root plasticity to C and N availability and whether these responses might be mediated by changes in shoot-to-root auxin transport. These questions were based on two main aspects of autoregulation. First, many autoregulation mutants show nodulation under high N availability, suggesting that the autoregulation signal interacts with an N signal to regulate nodule number or density (Day et al., 1989). Furthermore, many autoregulation mutants show lateral root and other growth phenotypes even in mock-inoculated conditions, although it has never been tested systematically whether these phenotypes respond to N or C availability. Second, the autoregulation mutant *sunn-1* is deficient in the regulation of shoot-to-root auxin transport in response to rhizobia at the onset of autoregulation in *M. truncatula*, and *sunn-1* nodule number can be reduced to wild-type levels by applying an auxin transport inhibitor at the shoot-root junction (van Noorden et al., 2006).

sunn-1 Affects Root Phenotypes in Response to N

Our results showed that the *sunn-1* mutant was characterized by lower root and shoot biomass, shorter lateral root length and taproot length, but similar lateral root density compared with the wild type at ambient-C and minus-N conditions. This was the case in inoculated as well as mock-inoculated conditions. As expected, *sunn-1* seedlings had an increased nodule number compared with the wild type, although the nodule number was not as much increased as in the soybean (*nts*), pea (*sym29*), and *L. japonicus* (*har1*) autoregulation mutants (Carroll et al., 1985a, 1985b; Sagan et al., 1995; Wopereis et al., 2000) or in *sunn-1*

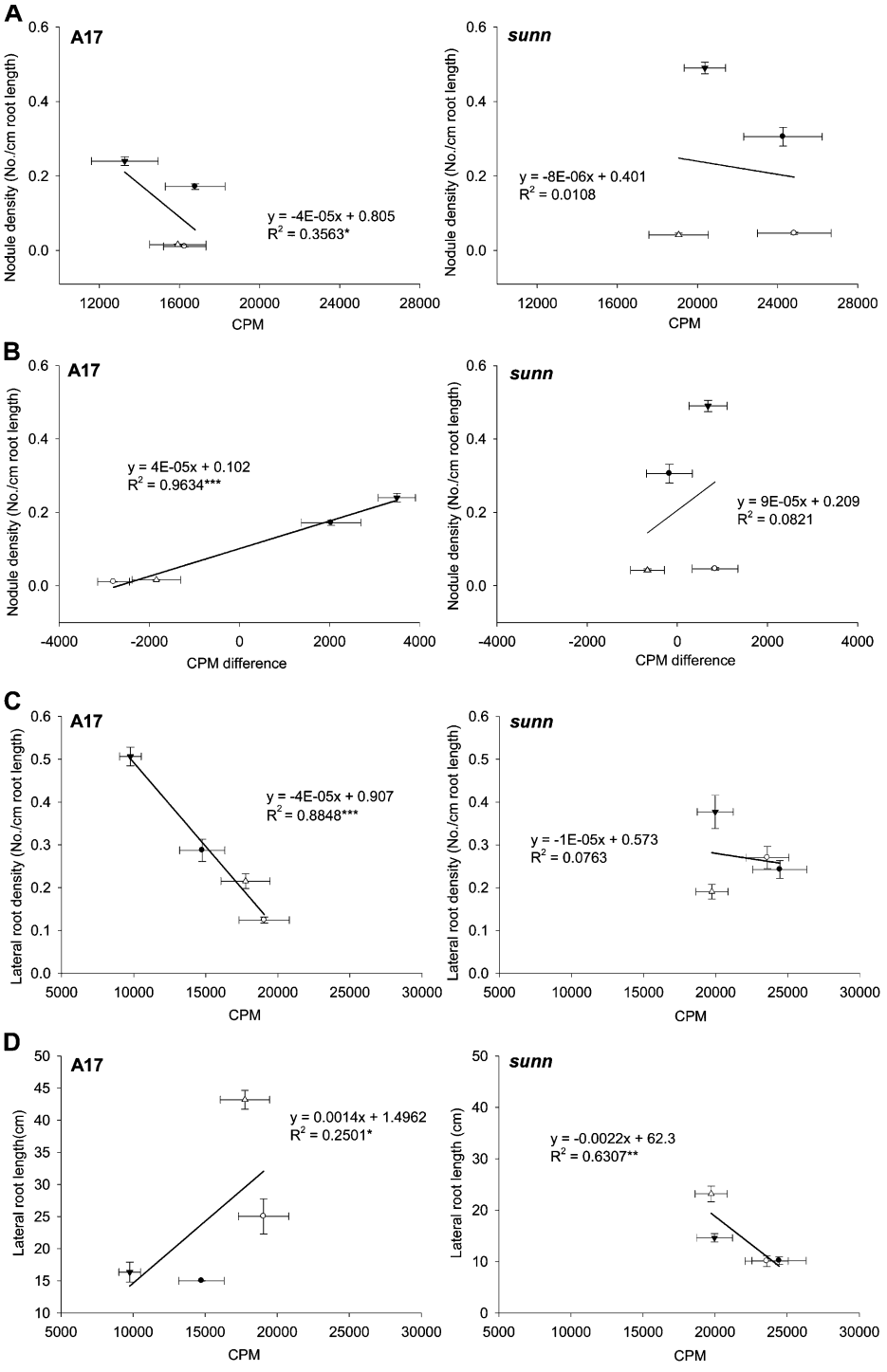

Figure 5. Correlation of auxin transport with root architecture phenotypes. A, Correlation of total auxin transport (as cpm) with nodule density in cv Jemalong A17 and *sunn-1*. B, Correlation of the cpm difference between inoculated and mock-inoculated roots with nodule density in A17 and *sunn-1* (i.e. positive values indicate more auxin transport in inoculated compared with mock-inoculated roots and vice versa for negative values). C, Correlation of total auxin transport with lateral root density in mock-inoculated seedlings of A17 and *sunn-1*. D, Correlation of total auxin transport with lateral root length in mock-inoculated seedlings of A17 and *sunn-1*. Seedlings were grown in ambient C (370 μL L^{-1} CO_2 [C_{370}]), high C (800 μL L^{-1} CO_2 [C_{800}]), minus N (0 mM nitrate [N_0]), and/or plus N (2.5 mM nitrate [$N_{2.5}$]). Inoculation with *S. meliloti* was performed on 5-d-old seedlings, and auxin transport measurements were made in 10-d-old seedlings. Significant correlations are marked with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ [calculated with unbalanced linear regression]). Data points indicate means ± SE with $n = 16$ to 20 for auxin transport measurements and $n = 24$ to 32 for nodule and lateral root measurements.

mutants grown under hydroponic conditions (Schnabel et al., 2010). Taproot length was previously found to be reduced in *sunn-1* (Penmetsa et al., 2003; Schnabel et al., 2010), *har1* (Wopereis et al., 2000), and *nts382* (Carroll et al., 1985a, 1985b) mutants. Lower plant dry weight was reported for *har1* (Wopereis et al., 2000) and soybean *nts* (Hansen et al., 1989) mutants, although the difference in plant mass between mock-inoculated *nts* and wild-type soybean plants was not significant (Day et al., 1986), in contrast to *sunn-1* plants. Lateral root density varies in different autoregulation mutants and was reported to be increased in *har1* (Wopereis et al., 2000) and *nts382* (Day et al., 1986) mutants but was unchanged in *sunn* mutants (Schnabel et al., 2005) compared with the wild type.

When supplied with altered [N] and [C], *sunn-1* mutants showed significantly different responses to the wild type, again under both inoculated and mock-

inoculated conditions. The root phenotypes of the wild type to added N were mostly as expected from other species: increased tissue [N] led to increased biomass (Carroll et al., 1985a; Hansen et al., 1992), a significant inhibition of nodule density (Streeter 1988), but no changes to taproot length (Drew et al., 1973; Zhang and Forde, 1998). Lateral root density in the wild type was reduced in response to increased shoot [N], which is thought to be a systemic effect (Zhang et al., 1999; Walch-Liu et al., 2006). However, high shoot N was also expected to inhibit lateral root emergence, resulting in less total root length (Zhang and Forde, 1998; Zhang et al., 1999; Walch-Liu et al., 2006), which was not observed in the wild type. Rather, increased lateral root elongation in the wild type in response to higher N resembled the responses of roots to a high localized supply of nitrate (Zhang and Forde, 1998). It is possible that these differences are due to the different nitrate concentrations used in the different studies, as inhibition of lateral root emergence in Arabidopsis was shown to require 10 mM or greater nitrate, while we only used 2.5 mM.

Inoculation only led to minor changes in biomass, lateral root density, and taproot length. Only lateral root length was significantly reduced in inoculated wild-type plants and in *sunn-1* plants under high C. The reason for this reduction is unknown but is unlikely to be due to resource reallocation to nodules, as it also occurred in plants forming almost no nodules. Interestingly, inoculation did not significantly increase tissue [N] in the plants growing in the absence of nitrate. Even though nodules were pink at the time of harvest at 20 d post germination, it is possible that N supply from nodulation was not sufficient to cover the plant's N needs, as reported previously for *M. truncatula* (Moreau et al., 2008).

Responses to increased C were generally not as pronounced as those to increased N and mainly resulted in enhanced plant growth, as observed in other studies (Stitt and Krapp, 1999; Fischinger et al., 2010). As expected, increased C also reduced tissue [N], which could be due to a dilution effect by increased C assimilation and by a decrease in N uptake under high CO_2 concentrations (Taub and Wang, 2008).

sunn-1 plants showed a smaller increase in biomass in response to increased C and N supply than the wild type, a smaller reduction in lateral root density, a smaller increase in lateral root length, but a decrease in taproot length in response to high N. However, in contrast to other autoregulation mutants, including *sunn-4*, *sunn-1* mutants still showed significant reductions in nodule number and density in response to increased N supply, and this is consistent with a previous study (Schnabel et al., 2010).

When taking into account the tissue [C] and [N], we showed that *sunn-1* seedlings had consistently higher shoot and root [N] than the wild type, whereas [C] was fairly constant in both genotypes. Tissue [N] has not previously been reported for *sunn* mutants. In soybean *nts* mutants, N content and concentration were found to be elevated, although this was only measured under inoculated conditions (Day et al., 1986; Hansen et al., 1989). Overall, the *sunn-1* mutant does not seem defective in uptake of N (or C) but in "translating" tissue [N] into the appropriate phenotypes.

Correlations of tissue [C] and [N] with root phenotypes showed that it was specifically the [N], not the [C], that drove changes in root architecture in the wild type. Increased shoot (or root) [N] showed significant negative correlations with nodule density, lateral root density, and taproot length in cv Jemalong A17. *sunn-1* plants still showed significant negative correlations of tissue [N] with nodule density and taproot length, but not with lateral root density. Therefore, it appears that the *sunn-1* mutant has lost some of its plasticity in regulating lateral root density in response to [N].

Our next question was whether changes in root architecture in response to [N] in *sunn-1* mutants are controlled by shoot-to-root auxin transport.

Does Shoot-to-Root Auxin Transport Mediate Root Developmental Changes in Response to C or N Supply?

We found previously that shoot-to-root auxin transport was inhibited by inoculation of the root with rhizobia within 24 h, corresponding to the time of onset of autoregulation under those conditions in wild-type *M. truncatula*. The *sunn-1* mutant showed elevated auxin transport from the shoot to the root compared with the wild type, and auxin transport inhibition did not occur after inoculation (van Noorden et al., 2006). In addition, inhibition of shoot-to-root auxin transport with the synthetic auxin transport inhibitor naphthylphthalamic acid reduced nodule number in *sunn-1* plants compared with the wild type (van Noorden et al., 2006). This led us to hypothesize that shoot-to-root auxin transport could be one of the autoregulation signals that control nodule number.

Here, we tested whether shoot-to-root auxin transport responds to N and C supply and whether this correlates with nodule density as well as lateral root density and elongation. It had been hypothesized that a systemic N signal could control lateral root emergence through shoot-to-root auxin transport, with the prediction that nitrate would reduce auxin transport from shoot to root, thus preventing lateral root emergence (Forde, 2002; Walch-Liu et al., 2006). In the wild type, shoot-to-root auxin transport was significantly affected by [N] but not by [C]. However, we found a significant increase in auxin transport with increasing shoot [N]. This led to a significant negative correlation between auxin transport and nodule and lateral root density but a positive correlation with lateral root length. Auxin transport measurements from shoot to root in maize showed that a 0.5 mM nitrate treatment reduced auxin transport, and this was correlated with increased lateral root length and no change to lateral root density (Liu et al., 2010). However, that study measured auxin after 1 h of nitrate treatment, while we measured auxin transport after 10 d, at a time when

lateral roots started to emerge. Another study in maize demonstrated that root indoleacetic acid (IAA) content and IAA concentration in phloem sap were reduced by the application of at least 5 mM nitrate, but this was correlated with decreased lateral root length, while lateral root density was not affected (Tian et al., 2008). In Arabidopsis, application of a synthetic auxin transport inhibitor above the site of nitrate treatment prevented the enhancement of lateral root length in response to nitrate, strengthening the idea that shoot-to-root auxin transport in response to N can influence lateral root length (Guo et al., 2005). The detailed relationship between nitrate treatment, auxin transport, and lateral root density and elongation might depend on plant species, time of measurement, nitrate concentration, and method of nitrate application. Our finding that auxin transport was strongly negatively correlated with lateral root density suggests that auxin concentration in the root might be superoptimal for lateral root initiation or early emergence in *M. truncatula*. This seems counterintuitive, as external auxin application usually increases lateral root number (Wightman et al., 1980), but it agrees with detailed measurements of auxin gradients in the root, which show that lateral root founder cells are established at sites of auxin minima (Dubrovsky et al., 2011).

We also found a negative correlation between nodule density and auxin transport. Again, this was unexpected, because higher auxin transport in *sunn-1* mutants is correlated with higher nodule density. However, as for lateral root initiation, it might be the establishment of local auxin gradients, rather than total auxin transport or content in roots, that determines the optimum conditions for nodule initiation. The local inhibition of auxin transport by rhizobia, which transiently establishes local auxin minima in the root before nodule initiation (Mathesius et al., 1998) and which also occurs in *sunn-1* mutants (van Noorden et al., 2006), might be a critical local control point for nodule initiation. It would be interesting in the future to find out in detail how shoot-to-root auxin transport interacts with local auxin gradients in the root.

In *sunn-1* plants, auxin transport was constitutively higher than in wild-type plants, and the amount of auxin transported did not respond to inoculation, confirming previous results (van Noorden et al., 2006). The interesting new finding was that in *sunn-1* mutants, auxin transport did not respond to shoot [N] as in the wild type. This suggests that *SUNN* is one of the regulators "translating" a systemic N signal into changes in long-distance auxin transport. Thus, the correlation between lateral root and nodule density with auxin transport was abolished in *sunn-1* mutants. This finding suggests two new hypotheses. First, nodule density seems not to be regulated primarily by the total amount of shoot-to-root auxin transport in response to [N], because while *sunn-1* mutants showed a strong correlation of nodule density with tissue [N], like the wild type, auxin transport in *sunn-1* was not correlated with nodule density in response to [N]. It is likely that in *sunn-1* mutants, local inhibition of nodulation by nitrate in the root overrides a defective autoregulation control. In this respect, *sunn-1* mutants might be an exception to the rule that most autoregulation mutants, including other *sunn* mutant alleles, still nodulate in the presence of nitrate. The local regulation of nodulation by nitrate is supported by the finding that nitrate induces a CLE-like peptide in soybean, GmNIC1, that probably interacts with NARK in the root to inhibit nodulation (Reid et al., 2011a). Another local regulator of nodulation could be NIP/LATD, a putative nitrate transporter expressed in roots and nodules that affects meristem activity in nodules and lateral roots (Bright et al., 2005; Yendrek et al., 2010).

Second, lateral root density seems likely to be under the control of shoot-to-root auxin transport in response to [N], because in the wild type, lateral root density was strongly dependent on shoot [N] and strongly correlated with auxin transport. This relationship appears to be under the control of *SUNN*, because *sunn-1* mutants failed to show a significant correlation between shoot [N] and lateral root density and between lateral root density and auxin transport. Therefore, we hypothesize that *SUNN* regulates lateral root development in response to tissue [N] systemically by the modulation of shoot-to-root auxin transport (Fig. 6).

CONCLUSION

Developmental plasticity toward changes in the C and N supply enables plants to maximize N capture while minimizing the costs associated with increased growth. Increasing N use efficiency in plants has focused mainly on improved N use uptake, transport, and metabolism (Garnett et al., 2009). The identification of genes that act in the root to sense N and subsequently regulate lateral root outgrowth, including auxin response genes, has suggested a local regulation of root plasticity toward N by auxin (Zhang

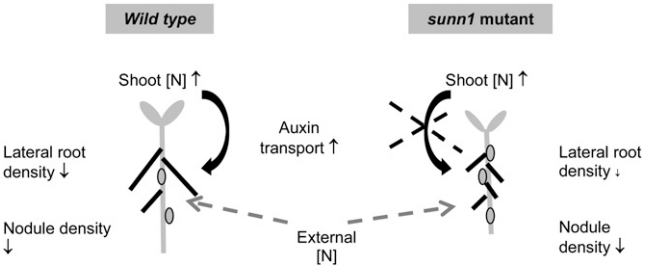

Figure 6. Model for the role of *SUNN* in lateral root and nodule development in response to tissue N concentrations. Our model hypothesizes that increasing shoot N concentration increases shoot-to-root auxin transport in the wild type but not in *sunn-1*. This is correlated with a reduction in lateral root density in the wild type but not in *sunn-1*. Nodule density reduces with increased [N] in both genotypes, suggesting a local inhibition of nodule initiation in the root in response to external N that is not correlated with total shoot-to-root auxin transport.

and Forde, 1998; Zhang et al., 1999; Krouk et al., 2010). However, so far, very few genes have been identified that translate shoot N status into appropriate changes in root architecture. Our results suggest that the *SUNN* gene may mediate changes in shoot N status into altered lateral root development by controlling shoot-to-root auxin transport. Therefore, *SUNN* could help in our understanding of N use efficiency at the level of developmental responses to N.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Medicago truncatula* 'Jemalong A17' (wild type) or *sunn-1* mutants were scarified with sandpaper, surface sterilized with 6% (w/v) sodium hypochlorite for 20 min, washed eight times in sterile distilled water, and transferred to 1% (w/v) water agar plates. After a 2-d period in the dark at 4°C, plates were transferred to 28°C overnight to germinate. Seedlings of approximately 1 cm in length were transferred to 15-cm-diameter petri dishes containing Fåhraeus medium (Fåhraeus, 1957). Fåhraeus plates contained either 2.5 mM KCl (with no further N source) or 2.5 mM KNO_3 as an N source. Each plate carried six to eight seedlings. Each treatment was replicated four times independently. Plates were kept vertical, the bottom one-third of each plate was sealed with Nescofilm, and the sides were covered for two-thirds with black paper. A sterilized aluminum foil spacer was placed between the lid and the petri dish to allow for air exchange. Plates were incubated in a growth chamber supplied with either 370 μL L^{-1} ambient CO_2 or 800 μL L^{-1} elevated CO_2. All treatments were randomized for their position in the growth chamber.

CO_2 concentration inside the petri dishes was measured using an infrared CO_2 analyzer (model LI-6251; Li-Cor).

The other conditions were consistent for all seedlings, with a temperature of 21°C, a light intensity of approximately 100 μmol m^{-2} s^{-1}, and 16 h of light per day. Five days after germination, the roots were inoculated with 5 μL of *Sinorhizobium meliloti* at the zone of emerging root hairs. *S. meliloti* strain 1021 was grown in liquid Bergensen's modified medium (Rolfe et al., 1980) at 28°C overnight and diluted with sterile water to an optical density at 600 nm of 0.1. As a control, the roots were inoculated with an equivalent volume of diluted Bergensen's modified medium.

Long-Distance Auxin Transport Measurement

Auxin transport was measured at 24 h (6-d-old seedlings) and 120 h (10-d-old seedlings) after inoculation with *S. meliloti*. Sixteen to 20 seedlings from each treatment were supplied with 12 pmol of [^{3}H]IAA (Amersham Bioscience; specific activity of 850 GBq $mmol^{-1}$) in 2 μL of ethanol that was applied between the cotyledons of the seedling. Seedlings were incubated vertically for 3 h in the dark and then cut into eight root segments of 5 mm length each, starting at the root-shoot junction. Each was placed in a scintillation tube with 3 mL of scintillation fluid (Perkin-Elmer). Samples were shaken overnight before being analyzed in a Beckman LS6500 scintillation counter (Beckman Instruments).

Root Morphology Measurement

At 20 d after germination, the numbers of first-order lateral roots and nodules were counted with a stereomicroscope. Root lengths were determined with an Epson 1680 modified flatbed scanner and WINRhizo software (Régent Instruments). Roots were placed in the Plexiglas tray in a shallow depth of water and scanned at 400 dots per square inch. Scans were first imported into Adobe Photoshop 7.0, and images were then analyzed with WINRhizo software for root length.

C and N Measurement

At harvest, the dry mass (oven dried at 70°C for 48 h) of shoot and root was recorded. Total C and N concentrations of shoot or root were determined using a Europa 20-20 isotope ratio mass spectrometer (Europa Scientific Instruments).

Statistical Analysis

Sample means and SE values were calculated for all parameters for each sampling date. ANOVA, residual maximum likelihood, and linear regressions were calculated using Genstat for Windows (version 11.1). Residual maximum likelihood was calculated when the data were unbalanced. The probability of a significant difference was set at $P < 0.05$.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. C and N contents of wild-type and *sunn-1* seedlings.

Supplemental Figure S2. Taproot length of cv Jemalong A17 and *sunn-1* seedlings.

Supplemental Figure S3. Correlations of shoot with root N concentration and of C concentration with nodule and lateral root density.

Supplemental Figure S4. Shoot-to-root auxin transport in response to inoculation and C and N treatments.

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