Defective Long-Distance Auxin Transport Regulation in the *Medicago truncatula super numeric nodules* Mutant[1][W]

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Long-distance auxin transport was examined in *Medicago truncatula* and in its supernodulating mutant *sunn* (*super numeric nodules*) to investigate the regulation of auxin transport during autoregulation of nodulation (AON). A method was developed to monitor the transport of auxin from the shoot to the root in whole seedlings. Subsequently, the transport was monitored after inoculation of roots with the nodulating symbiont *Sinorhizobium meliloti*. The *sunn* mutant showed an increased amount of auxin transported from the shoot to the root compared to the wild type. The auxin transport capacity of excised root segments was similar in wild type and *sunn*, suggesting that the difference in long-distance auxin transfer between them is due to loading in the shoot. After inoculation, wild-type seedlings showed decreased auxin loading from the shoot to the root; however, the *sunn* mutant failed to reduce the amount of auxin loaded. The time of reduced auxin loading correlated with the onset of AON. Quantification of endogenous auxin levels at the site of nodule initiation showed that *sunn* contained three times more auxin than wild type. Inoculation of *sunn* failed to reduce the level of auxin within 24 h, as was observed in the wild type. We propose a model for the role of auxin during AON of indeterminate legumes: 1) high levels of endogenous auxin are correlated with increased numbers of nodules, 2) inoculation of roots reduces auxin loading from the shoot to the root, and 3) subsequent reduction of auxin levels in the root inhibits further nodule initiation.

Legumes have the ability to form a symbiotic relationship with soil bacteria called rhizobia. This interaction leads to the formation of nitrogen-fixing root nodules, which is a result of an exchange of signals between the symbiotic partners. The initiation of nodules by rhizobia is regulated by lipochitin oligosaccharides (Dénarié et al., 1996) and involves the reinitiation of the cell cycle in the cortical and pericycle cells of the host root. In indeterminate legumes, nodules are initiated in the inner cortex, in contrast to determinate legumes, where nodules typically start to develop in outer cortical cells (Hirsch, 1992). The nodule primordia start to differentiate and form a mature nodule that is inhabited by rhizobia. *Medicago truncatula* and *Lotus japonicus* have been chosen as model legume plants for the legume-rhizobia interaction forming indeterminate (Barker et al., 1990;

Cook, 1999) or determinate nodules (Handberg and Stougaard, 1992; Udvardi et al., 2005), respectively. In most legumes, the initiation of nodules is restricted to a narrow susceptible zone of the root near the zone of emerging root hairs (Bhuvaneswari et al., 1981). The number of nodules is tightly regulated by the host plant and established nodules suppress the formation of new nodules on the same plant (Caetano-Anollés and Bauer, 1988; Caetano-Anollés and Gresshoff, 1991). This mechanism of restricting nodule numbers is called autoregulation of nodulation (AON). In indeterminate legumes, autoregulation blocks the formation of new nodule primordia, whereas in determinate legumes, autoregulation stops already initiated primordia from developing into a nodule (Caetano-Anollés and Gresshoff, 1991). Removal of already established nodules and root tips enables the formation of new nodules (Nutman, 1952; Caetano-Anollés et al., 1991). The number of nodules is also regulated in response to environmental signals, e.g. nitrate (Streeter, 1988) and the plant hormone ethylene (Penmetsa and Cook, 1997).

Several supernodulating mutants, which have lost the ability to autoregulate nodule numbers, have been described. First to be discovered was the supernodulating soybean (*Glycine max*) *nts* (*nitrate-tolerant supernodulating*) mutant (Carroll et al., 1985a, 1985b). In *M. truncatula*, the *sunn* (*super numeric nodules*) mutant was shown to form 10 times more nodules than the wild-type plant (Penmetsa et al., 2003). Supernodulating mutants in other legumes, like the *L. japonicus har1*

(Krusell et al., 2002), the pea (*Pisum sativum*) *sym29* (Krusell et al., 2002), as well as the soybean *nts* (Searle et al., 2003) and *M. truncatula sunn* (Schnabel et al., 2005) mutants, have all been reported to have mutations in a Leu-rich repeat receptor-like kinase (LRR-RLK). Grafting of these supernodulating plants shows that the autoregulation signal is derived from the shoot and inhibits nodule formation in the root (Delves et al., 1986; Krusell et al., 2002; Penmetsa et al., 2003). So far, the autoregulation signal itself has not been identified, and it is not known how a mutation in an LRR-RLK gene mediates AON.

The phytohormone auxin has long been suspected of playing an important role in the initiation and development of nodules, since Thimann (1936) observed elevated levels of auxin in pea nodules. In plants, auxin occurs predominantly in the form of indole-3-acetic acid (IAA). The highest IAA concentrations are found in young tissues, such as young leaves and in the shoot apical meristem, suggesting that IAA is mainly synthesized in these locations. However, it is also synthesized in the root (Ljung et al., 2005). Auxin is transported in a basipetal direction (Baluska et al., 2005) in the stem and in an acropetal direction in the root. This transport is known as polar auxin transport (PAT). PAT is mediated by at least two transport systems including the auxin importer, AUX1, and a class of auxin export facilitators (PIN [pin-formed] proteins). PIN proteins are asymmetrically distributed on either the basal or apical side of cells, and it has been suggested that this controls the polarity of auxin transport. At the root tip, auxin transport reverses to a basipetal direction in the epidermal and outer cortical cells in Arabidopsis (*Arabidopsis thaliana*; Blilou et al., 2005). In addition, it is likely that other proteins interacting with PIN have important roles in maintaining the polarity of auxin transport, including multidrug resistance proteins (Noh et al., 2001; Geisler et al., 2005; Terasaka et al., 2005). Auxin transport can be inhibited by synthetic auxin transport inhibitors, including *N*-(1-naphthyl)phthalamic acid (NPA), which is thought to interfere with the intracellular cycling of the PIN proteins between the plasma membrane and endosomal vesicles (Geldner et al., 2001; for full review, see Blakeslee et al., 2005).

A number of experiments suggest that rhizobia manipulate auxin transport. The application of synthetic PAT inhibitors, which interfere with the hormone balance in the root, can induce pseudonodule structures on the root (Allen et al., 1953; Hirsch et al., 1989; Wu et al., 1996). PAT inhibitors are also sufficient to induce some of the nodulin genes inside pseudonodules, including *ENOD2* and *ENOD12* (Hirsch et al., 1989; Scheres et al., 1992; Wu et al., 1996). Direct measurements of auxin transport using radiolabeled auxin showed that rhizobia locally inhibit acropetal auxin transport capacity in vetch (*Vicia sativa*) roots within 24 h after inoculation (Boot et al., 1999). In addition, the expression of an auxin-responsive promoter (*GH3*) was reduced acropetally from the inoc-

ulation site, between 12 and 24 h following rhizobia inoculation or ballistic microtargeting of Nod factors (Mathesius et al., 1998). This was followed by an apparent increase in auxin accumulation at the site of nodule initiation in the inner cortex. In contrast, in the determinate legume *L. japonicus*, no auxin transport inhibition could be measured after inoculation (Pacios-Bras et al., 2003). However, an increase of *GH3* expression was still located in the nodule initials, in this case, in the outer cortical cells, suggesting that high auxin levels are required for nodule initiation. Moreover, it has been suggested that the expression of the AUX-1-like protein LAX in developing *M. truncatula* nodule primordia is important for a continuous flow of auxin into the forming primordium (de Billy et al., 2001). It is therefore likely that auxin transport regulation is part of the process leading to nodule initiation.

Since auxin is a plant hormone transported from the shoot to the root, and because it appears to be an important regulator of nodule initiation, it has been suggested that auxin is part of the autoregulation control of nodulation. Gresshoff (1993) proposed the hypothesis that in soybean, a determinate legume, initiated nodules send a signal to the shoot, which then causes a burst of auxin translocation from the shoot to the root, subsequently preventing the formation of new nodules. This hypothesis is known as the auxin burst control hypothesis and this control mechanism is thought to have been lost in the supernodulating *nts* mutant.

The *M. truncatula sunn* mutant has a short root phenotype, and expression studies showed that the auxin response gene *GH3* was expressed at higher levels in *sunn* than in the wild type following inoculation with *Sinorhizobium meliloti* (Penmetsa et al., 2003). This indicated that the *sunn* phenotype could also be the result of an auxin defect. In this study we aimed at determining whether auxin transport is involved in AON by comparing local and long-distance regulation of auxin transport, and auxin levels in the wild type and in the *sunn* mutant seedlings. Our results show that the *sunn* mutant has increased auxin transport and auxin content and that long-distance auxin transport regulation by rhizobia is defective.

RESULTS

Growth Characteristics of Wild-Type and *sunn* Seedlings

To confirm the supernodulating phenotype of *sunn* under our growth conditions, we grew wild-type and *sunn* seedlings on agar plates in the presence and absence of its symbiont, *S. meliloti*. Seedlings of the *sunn* mutant grew to approximately 70% of the length of the wild type 4 d after germination. This was due to shorter root and hypocotyl lengths ($P < 0.001$ and $P < 0.05$, respectively; Fig. 1A). Examination of cortical cell length in the mature root zone of 4-d-old seedlings (2 cm from the root tip) showed that *sunn* cortical cells were only 85% of the wild-type cell length ($P < 0.001$;

Figure 1. Phenotypes of *M. truncatula* wild type and *sunn*. A, Length of 4-d-old seedlings, and the length of the roots and hypocotyls separately (*n* = 60). B, Cortical cell length in the mature root zone of 4-d-old seedlings (*n* = 46–50). C, Nodule number of 21-d-old plants inoculated with *S. meliloti* (*n* = 15). Results are means ± SE. Values denoted with an * or *** are significantly different from that of the wild type at the 0.05 and <0.001 level, respectively (Student's *t* test). D, Time point of AON. Numbers of nodules that formed 21 d postinoculation in a root segment corresponding to different time points after inoculation. Results are means (*n* = 72 for wild type and 58 for *sunn*). LSDs at the 0.05 level (ANOVA) are 0.29 and 0.71 for wild type and *sunn*, respectively.
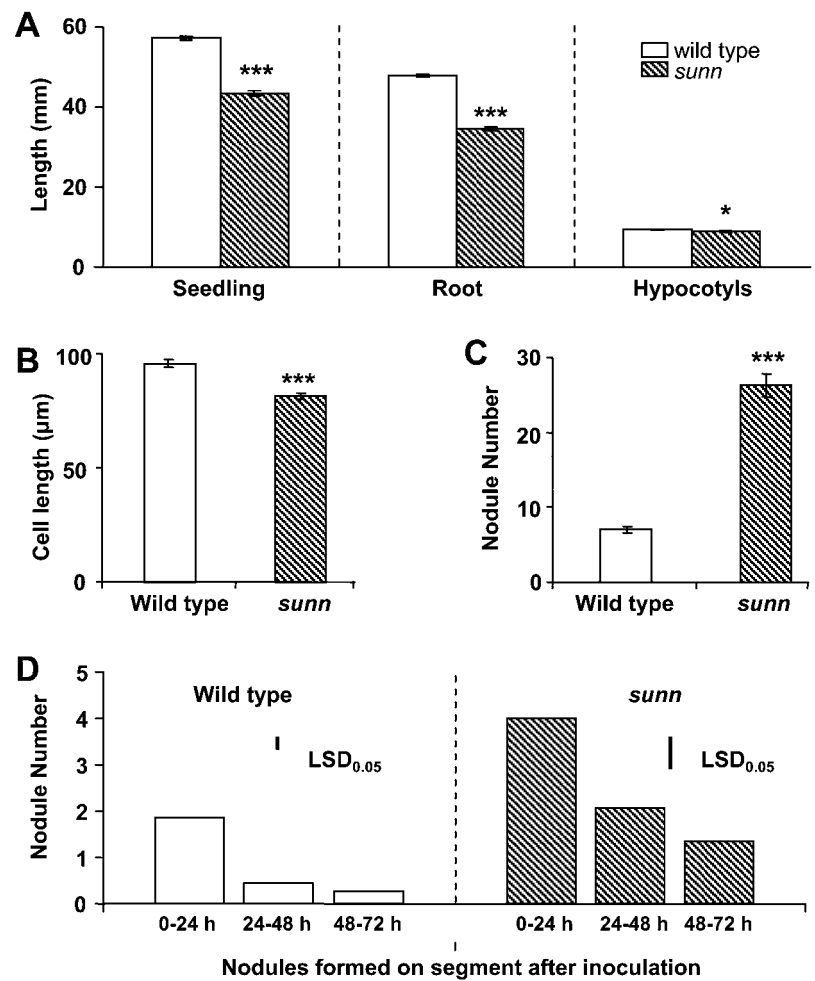
Fig. 1B). Inoculated roots of wild-type seedlings formed an average number of seven nodules per root, whereas *sunn* seedlings formed an average of 26 nodules per root within 3 weeks after inoculation (Fig. 1C). These results were similar to those reported previously for *sunn* (Penmetsa et al., 2003; Schnabel et al., 2005), which were obtained under aeroponic growth conditions rather than on plates.

Time Point of AON

To determine the time point at which the autoregulation signal starts to inhibit nodule formation, wild-type plants were inoculated with *S. meliloti*, and the position of the root tip was marked. New nodule numbers on the primary root were significantly reduced (*P* < 0.001) in the root segment corresponding to the time point between 24 and 48 h, and 48 and 72 h after inoculation, as compared to the 0- to 24-h segment (Fig. 1D). Beyond the 72-h time point, more nodules were formed. However, these later nodules were spread out along the root (data not shown). These results show that nodule formation is inhibited most strongly from 24 h after initial inoculation of the

seedling roots; however, some nodules can be formed afterward. In *sunn*, a reduction in nodule numbers was also observed at the same time points as in the wild type (*P* < 0.001; Fig. 1D), although this reduction in numbers was not as pronounced as in the wild type. These results agree with observations by Penmetsa et al. (2003) who found that the supernodulation response in *sunn* was transient.

Response of Wild Type and *sunn* to Auxin and Auxin Inhibitors

To test whether auxin is directly involved in nodulation, plants were grown on the known auxin action inhibitor, *p*-chlorophenoxyisobutyric acid (PCIB; Oono et al., 2003); the auxin transport inhibitor, NPA; and on the auxins IAA, naphthyl-acetic acid (NAA), or 2,4-dichlorophenoxy acetic acid (2,4-D). Plants grown on the auxin action inhibitor, PCIB, at concentrations between 10^{-5} and 10^{-8} M showed a significant (*P* < 0.001) reduction in nodule numbers (Fig. 2A; data not shown); *sunn* and wild type showed similar responses.

When plants were grown on plates containing either the auxin transport inhibitor, NPA, or the auxins IAA,
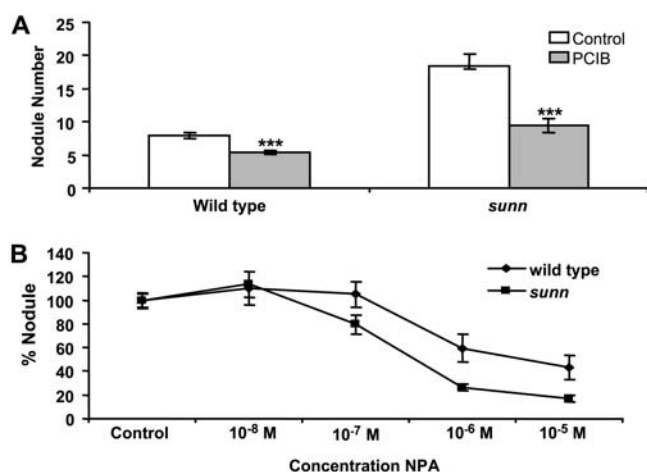
Figure 2. Nodulation response to auxin and auxin inhibitors. A, Nodule numbers in 21-d-old plants inoculated with *S. meliloti* grown on media containing 10^{-5} M PCIB. Results are means ($n = 28$) ± SE. PCIB results denoted with an *** are significantly different from that of the controls at the <0.001 level (Student's *t* test). B, Relative nodule numbers from 21-d-old plants inoculated with *S. meliloti* grown on media containing different concentrations of NPA. Results are means ($n = 12–15$) ± SE.

NAA, or 2,4-D at concentrations between 10^{-5} and 10^{-7} M (10^{-8} M for NPA), there were no significant differences in the relative growth rate of the roots between wild type and *sunn*. In both cases, root growth was inhibited with increasing concentrations of NPA or auxins (data not shown). Nodule numbers also decreased with increasing NPA concentrations; however, the relative inhibition of nodule numbers in *sunn* was significantly greater than in wild type (Fig. 2B). In response to added auxins, at higher concentrations, nodule numbers decreased similarly in *sunn* and wild type. However, some low concentrations of IAA (below 10^{-8} M) promoted nodulation in the wild type (Supplemental Fig. 1; data not shown).

When NPA was applied locally just below the cotyledons to reduce auxin transfer from shoot to root, nodule numbers in the wild type were not significantly reduced, but *sunn* showed a significant ($P < 0.001$) reduction in nodule numbers (Fig. 3). The reduction in nodule initiation was evident both before and after 48 h after inoculation.
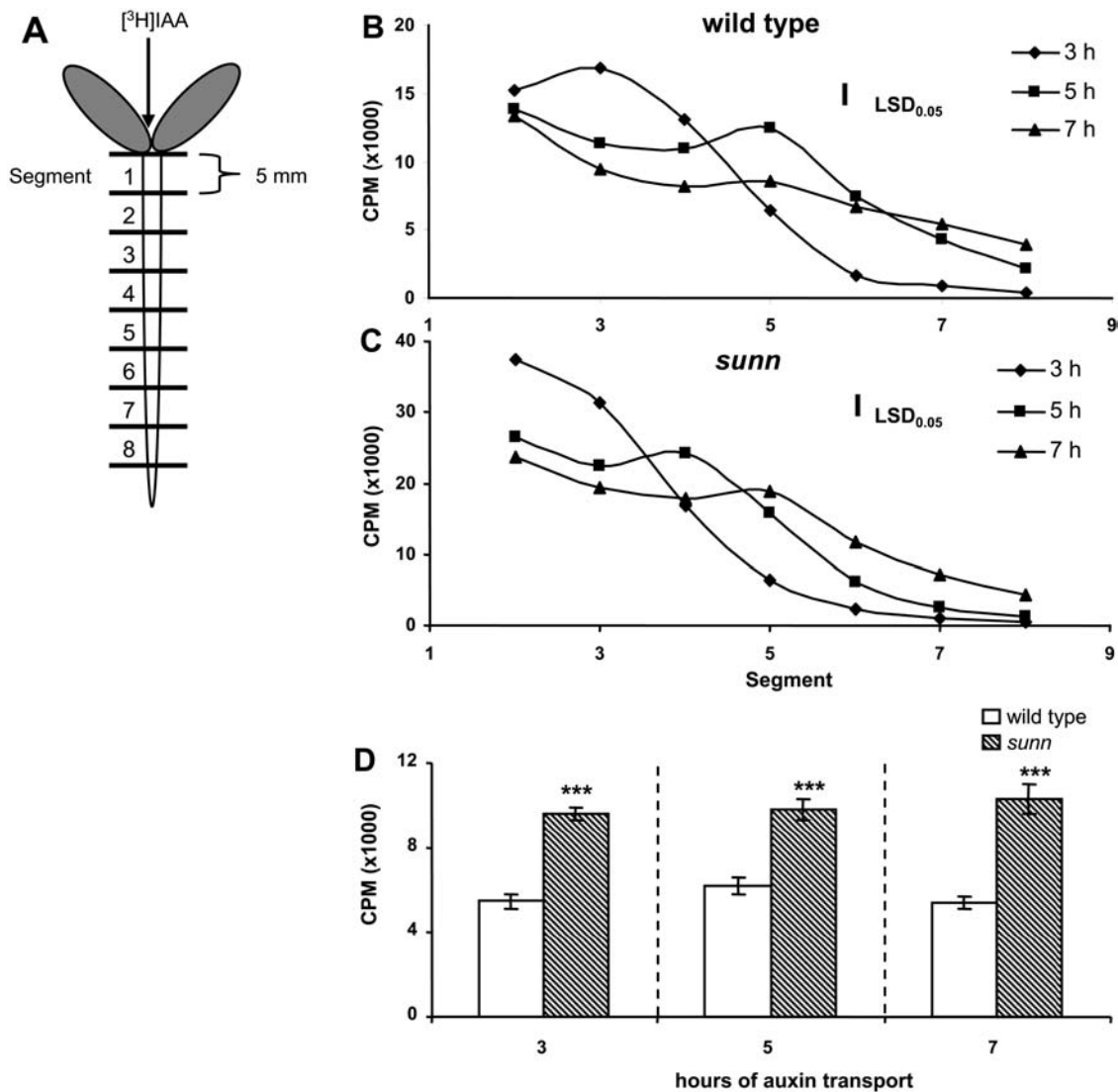
Long-Distance Auxin Transport Measurements

To measure the PAT from the shoot to the root (long-distance auxin transport), we adjusted a method (Beveridge et al., 2000) to monitor the flow of [³H]-labeled IAA in whole *M. truncatula* seedlings (see Fig. 4A). [³H]IAA was applied at the shoot apex between the cotyledons and moved down the root within 3 h. To verify that this experiment measured PAT in the roots, we applied the synthetic auxin transport inhibitor, NPA, in the agar plates at various concen-

trations from 10^{-8} to 10^{-5} M 18 h before the start of auxin transport measurements. In both wild-type and *sunn* roots, the speed at which the radiolabeled auxin moved down the root and the total amount of radioactivity transported down the root were significantly ($P < 0.001$) reduced by NPA (Supplemental Fig. 2). This experiment confirmed that changes in auxin transport by a known auxin transport inhibitor can be detected in our system and that both wild type and *sunn* respond to NPA. To test whether the radiolabeled peak recovered from the roots after 3 h of transport was still radiolabeled IAA, roots were extracted, the extracts separated by HPLC, fractions collected every minute, and each fraction was tested for radioactivity. Wild-type and *sunn* plants showed a similar pattern of radioactivity after HPLC. In both cases, the main radioactive peak recovered after HPLC corresponded to the retention time of an IAA standard (data not shown). These experiments confirmed that our method is capable of measuring PAT in *M. truncatula* seedling roots. However, we observed that similar experiments performed on different days varied in the total radioactivity measured in the roots. Relative changes in auxin transport amount and speed did not vary. The variation was most likely due to individual batches of radiolabeled auxin and changes in seed germination. Thus, no direct comparisons can be made for total counts between experiments from different days.
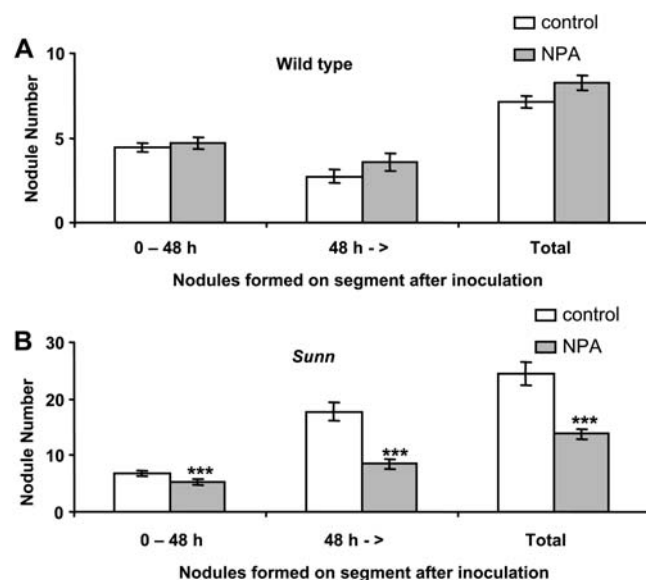
Figure 3. Nodulation response to local auxin transport block, below the cotyledon. Numbers of nodules that formed 21 d postinoculation in a root segment corresponding to different time points after inoculation. A, Nodules formed in wild-type control and NPA-treated plants. Results are means ($n = 26$) ± SE. B, Nodules formed in *sunn* control and NPA-treated plants. Results are means ($n = 36$) ± SE. NPA results denoted with an *** are significantly different from that of the control at the <0.001 level (Student's *t* test).

Figure 4. Long-distance auxin transport experiments. A, Experimental design for measuring long-distance PAT in whole seedlings. Radiolabeled auxin is 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, are excised into scintillation fluid. Segment 1 is not taken into account for the analysis because of large variation of radioactivity due to diffusion from the site of application. B and C, PAT in intact 4-d-old wild-type and *sunn* seedlings after 3, 5, and 7 h of [3H]IAA transport. LSDs at 0.05 level (REML) are 1,629 and 3,112 for wild type and *sunn*, respectively ($n = 20$). D, Total transported [3H]IAA in wild-type and *sunn* seedlings after 3, 5, and 7 h of transport. Results show the sum of the total amount of radiolabeled auxin transported into segments 2 to 8 from the experiment in B and C and are means ± SE ($n = 20$). *sunn* values denoted with *** are significantly different from that of the wild type at the <0.001 level (Student's *t* test).

## Comparison of Long-Distance Auxin Transport in Wild Type and *sunn*

Long-distance auxin transport was compared between untreated wild-type and *sunn* seedlings. Auxin transport was measured at 3, 5, and 7 h after application of [3H]IAA to the shoot apex. In wild-type roots, auxin was transported faster per centimeter of root than in the *sunn* seedlings (Fig. 4, B and C). The time it took for the maximum amount of the radiolabeled auxin to reach segment 5 of the roots was approxi-

mately 5 h in wild type and 7 h in *sunn*. Considering that the root length of *sunn* was only 70% of the wild type, the speed of auxin transport compared to the root length was similar in wild type and *sunn*. However, the total amount of auxin recovered in wild type compared to *sunn* roots was significantly lower ($P <$ 0.001; Fig. 4D). These results suggest that the main difference in auxin transport between wild type and *sunn* is the amount of auxin transported from the shoot apex into the roots.

Long-Distance Auxin Transport in
S. meliloti-Treated Plants

To test if inoculation of the roots had an effect on the long-distance auxin transport, 3-d-old *M. truncatula* root tips were flood inoculated with *S. meliloti*, and radio-labeled auxin was applied 1, 24, 48, and 72 h after inoculation. The radiolabeled auxin was then allowed to transport for 3 h. All experiments were repeated at least three times on different days with 16 to 20 seedlings

each. After 1 h of inoculation no significant differences in auxin transport could be found in either the wild-type or *sunn* seedlings compared to mock-inoculated roots (Fig. 5, A and C). After 24 h following inoculation, a significant ($P < 0.05$) reduction of the amount of [³H]IAA transported could be observed in the wild type (Fig. 5B). This reduction of auxin transport after 24 h correlated with the onset of autoregulation. However, the auxin was still transferred with the same speed as in the control
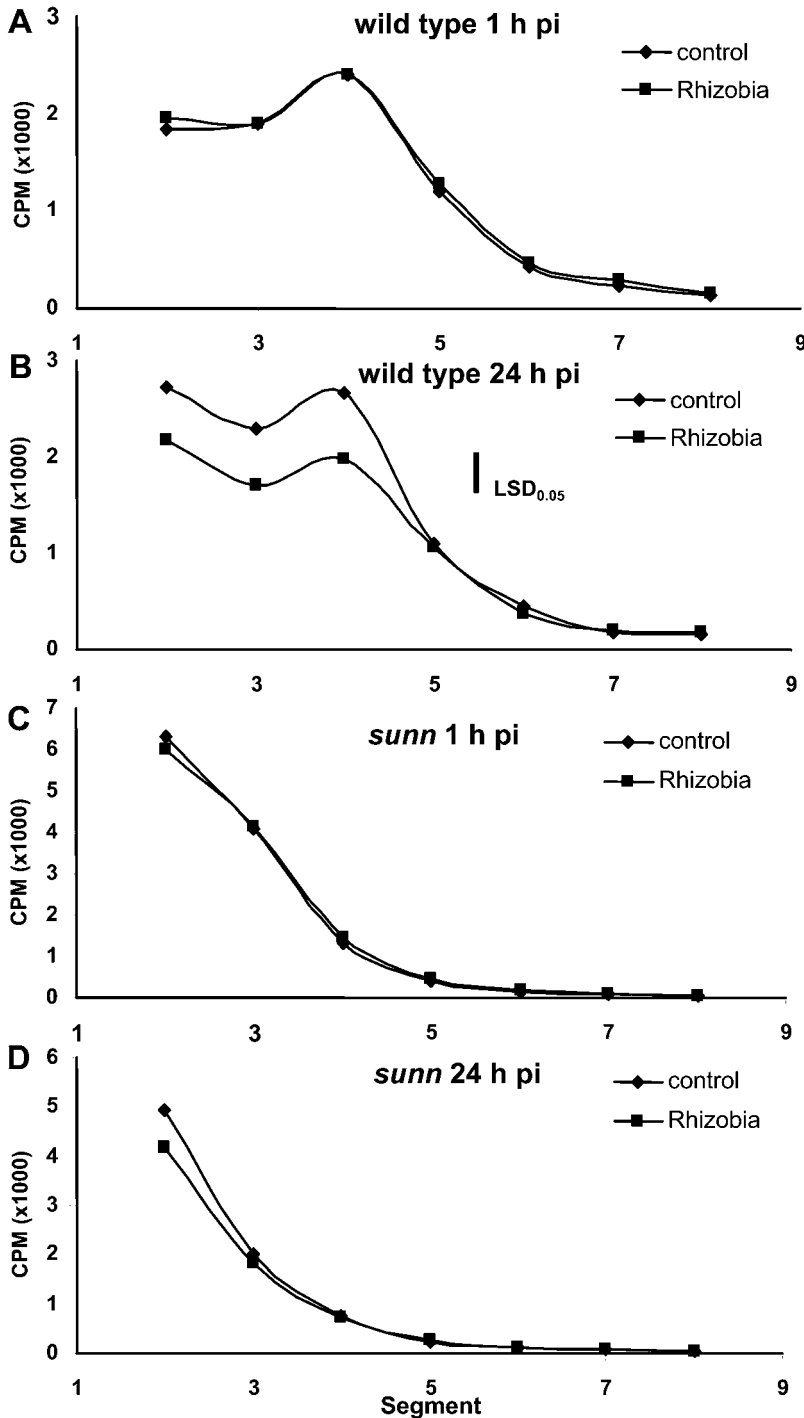
Figure 5. Long-distance PAT in whole wild-type and *sunn* seedlings after inoculation with *S. meliloti*. A and B, PAT in intact 4-d-old wild-type seedlings 1 and 24 h after inoculation with *S. meliloti* and after a subsequent 3-h period of [³H]IAA transport. LSD at 0.05 level (ANOVA) was 424, at 24 h after inoculation. C and D, PAT in intact 4-d-old *sunn* seedlings 1 and 24 h after inoculation with *S. meliloti* and after 3 h of [³H]IAA transport. The experimental set up was as shown in Figure 4A ($n = 16$–20).

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roots. There was no consistent reduction in auxin transport after 48 and 72 h (data not shown).

In the *sunn* mutant, no significant difference in the amount of transported [³H]IAA or auxin transport speed was found in response to inoculation with rhizobia at any time point (Fig. 5D).

Local Auxin Transport Measurements in Hypocotyls and Roots

To test whether the difference in radiolabeled auxin transported in wild type and *sunn* was due to a difference in the transport capacity of auxin between the hypocotyl and the root, we measured the amount of auxin transported from a donor block by an excised segment at the hypocotyl-root junction (set up as in Fig. 6, A and B) in the wild-type and *sunn* mutant. We

found no significant difference in the amount of radioactivity recovered from either root segments (Fig. 6C) or receiver blocks (data not shown) between *sunn* and wild type. This finding suggests that the difference in the amount of auxin loaded into the root in wild type and *sunn* is most likely to occur between the shoot apex and the top of the hypocotyl.

Local inhibition of auxin transport by rhizobia has been previously linked to initiation of nodules (Mathesius et al., 1998; Boot et al., 1999). To determine the local auxin transport capacity after inoculation, 4-d-old seedlings were spot inoculated with *S. meliloti* at the susceptible zone near the emerging root hairs (Fig. 6B). The auxin transport capacity was measured by placing a donor block on the excised root segment 1, 6, 12, and 24 h after inoculation and leaving it on the root for 18 h for the auxin to move into the root and


Figure 6. Local auxin transport experiments. A, Experimental design for measuring local capacity of auxin transport in excised root or hypocotyl segments. A block of agar containing radiolabeled auxin (donor block) is positioned on top of the excised root surface. An agar block without auxin (receiver block) is placed at the lower side of the segment. After 18 h of transport, the segment is cut into two 4-mm segments (below and above the star), and radioactivity of both root segments and in the receiver block is measured. The star indicates the inoculation site or the hypocotyl-root junction. B, Cartoon of a seedling showing the two sites at which local auxin transport was measured, the hypocotyl-root junction and the zone of emerging root hairs. C, Measurements of auxin transport at the hypocotyl-root junction. Results are means ± SE (*n* = 20) of the amounts of radioactivity that were recovered from segment 2. D, Auxin transport in root segments of wild type and *sunn* after inoculation. The roots were spot inoculated with *S. meliloti* at the zone of emerging root hairs, and auxin donor blocks applied 1 h after inoculation. Results are the means ± SE (*n* = 34–36) of the amounts of radioactivity that were recovered from segment 2. Values denoted with an * and ** are significantly different from that of the control-treated roots at the 0.05 and 0.01 levels, respectively (Student's *t* test).
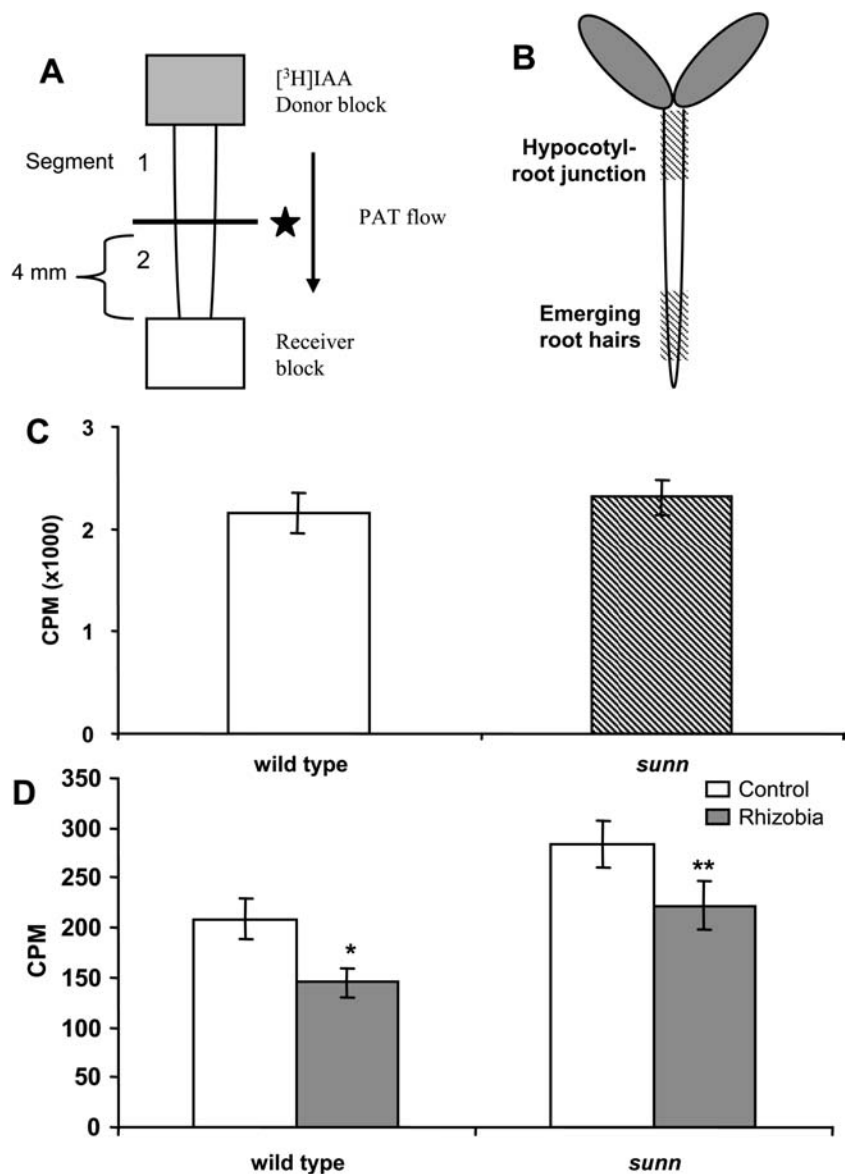
receiver block (Fig. 6A). As soon as 1 h after inoculation, the auxin transport capacity was significantly ($P < 0.05$) reduced in the wild-type and the *sunn* mutant, both showing an approximately 20% to 30% reduction in auxin transported (Fig. 6B); at 6 h after inoculation, the auxin transport capacity was still reduced in both the wild type and *sunn*. However, after 12 h of inoculation, no reduction in auxin transport capacity could be detected, and 24 h after inoculation the capacity for auxin transport was increased in the wild type and *sunn* (data not shown). The exact timing of the start of the reduction in auxin transport capacity and the following events is difficult to determine in this experimental design because of the 18-h time span necessary for the auxin transport measurements. Therefore, the onset of local auxin transport inhibition starts between 1 and 19 h following inoculation. However, the wild type and *sunn* followed the same trend in the local reduction of auxin transport capacity.

Endogenous IAA Levels

To test whether changes in auxin transport result in changes in auxin concentration in wild-type and *sunn* roots and shoots, IAA levels were quantified from whole roots and shoots in 4-d-old seedlings. Free endogenous IAA levels were significantly ($P < 0.05$) higher in the *sunn* roots and shoots than in the wild-
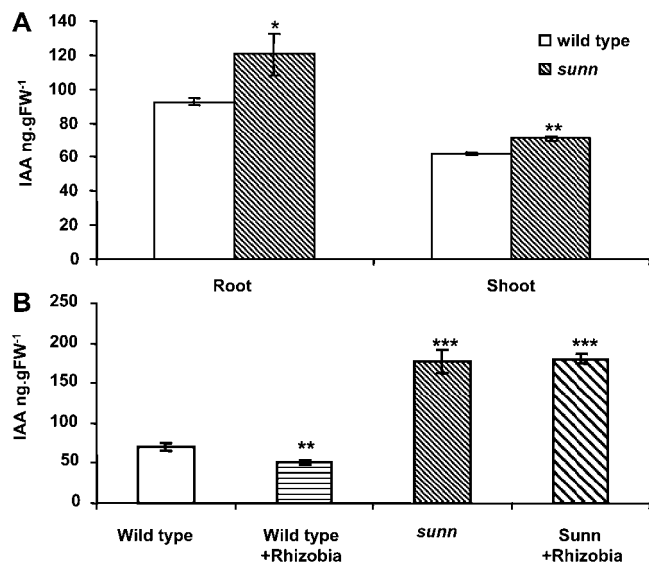
Figure 7. Endogenous auxin levels in wild type and *sunn*. A, Auxin levels in whole roots and shoots (including hypocotyls) of 4-d-old wild-type and *sunn* seedlings. Data are results of three pools of 40 roots ± SE. *sunn* values denoted with an * or ** are significantly different from that of the wild type at the 0.05 and 0.01 levels, respectively (Student's *t* test). B, Auxin levels in root segments (1 cm length) spanning the inoculation site 24 h after inoculation with *S. meliloti*. Data are results of three to four pools of 40 root segments ± SE. Values denoted with an * or *** are significantly different from that of the wild-type control at the 0.05 and <0.001 levels, respectively (Student's *t* test).

type roots and shoots (Fig. 7A). Roots were then inoculated in the zone of emerging root hairs with *S. meliloti*, and after 24 h, a segment of 1-cm length spanning the inoculation site was harvested. At this time point, the harvested segment corresponded to the young mature root zone. Auxin levels decreased significantly ($P < 0.05$) in the wild type after inoculation (Fig. 7B). In *sunn* roots, auxin levels in the zone of inoculation were approximately 3 times as high as in the wild type ($P < 0.001$), but were not significantly reduced after inoculation (Fig. 7B). Shoot auxin levels were not affected in wild type or *sunn* after inoculation (data not shown).

DISCUSSION

Is Auxin Involved in Nodulation?

Several reports in the literature suggested that auxin is involved in nodulation, including evidence that nodules contain elevated levels of auxin, that auxin response genes are differentially expressed during nodule initiation, that auxin transport is inhibited by rhizobia, and that auxin transport inhibitors can induce pseudonodules (for review, see Ferguson and Mathesius, 2003). Here we show that the auxin action inhibitor, PCIB, which was previously reported to inhibit the activation of auxin response genes (Aux/IAA genes) in Arabidopsis (Oono et al., 2003), also significantly reduces nodule numbers in *M. truncatula* (Fig. 2A). This result provides further evidence that auxin action is necessary for nodule formation. In addition, we found that auxin applied at low levels could promote nodulation, while high levels inhibited nodulation, suggesting that a narrow optimum of auxin concentration exists to stimulate that process. The fact that wild type and *sunn* showed similar relative changes in root growth and nodulation to the different auxins suggests that *sunn* does not have an altered response to auxin per se. The auxin transport inhibitor NPA also reduced nodule numbers (Fig. 2B); however, in this case *sunn* was more sensitive to NPA application than the wild type. An important question is therefore whether the action of auxin during nodulation is regulated through local and/or long-distance auxin transport and whether *sunn* has a defect in auxin transport.

Evidence for Local and Long-Distance Control of Auxin Transport during Nodulation

The hypothesis tested in this study was that the *sunn* phenotype was due to a direct or indirect defect in the regulation of auxin transport. To differentiate between local and long-distance regulation of auxin transport we developed a method to monitor PAT in *M. truncatula* seedlings. Using this method, we could determine the speed and the total amount of radiolabeled auxin transported from the shoot into and along the root. This is in contrast to previous methods for measuring local auxin transport regulation in legumes, where ei-

ther auxin responsive genes were used as an indirect measure of auxin localization in the root (Mathesius et al., 1998; Pacios-Bras et al., 2003) or auxin transport capacity was measured in excised root segments around the inoculation site (Boot et al., 1999; Pacios-Bras et al., 2003).

Using our long-distance method, we found that the speed of auxin transport was slower in the *sunn* mutant compared to the wild type (Fig. 4, B and C). However, the cells of *sunn* roots were shorter than in the wild type (Fig. 1B), and therefore the mutant had more cells per length of root. Since PAT is a cell-to-cell transport, the speed of auxin transport was approximately correlated with the number of cells through which the auxin was transported. Therefore, the relative speed (the time that is needed to get from the shoot to the root tip) was approximately the same as in the wild type. In addition to cell-to-cell PAT, auxin is also thought to travel in the phloem (Woodward and Bartel, 2005). To what extent the auxin measurements reflect phloem transport was not determined here. The observation that NPA strongly reduced the amount of auxin transported to the root suggests that the majority of auxin transport was due to cell-to-cell transport. The most striking difference in auxin transport between *sunn* and wild type was that the amount of auxin transported into the root was significantly higher in *sunn* compared to the wild type (Fig. 4D). This could be due to either higher auxin loading from the shoot to the root or a higher capacity for auxin transport along the root. To distinguish between those possibilities, we measured auxin transport capacity in excised segments spanning the hypocotyl-root junction and found that the capacity for the auxin transport at the hypocotyl-root junction was not altered in the *sunn* mutant (Fig. 6C). These results suggest that in the *sunn* mutant a higher amount of auxin is loaded into the auxin transport system, compared to the wild type, above (acropetal to) the hypocotyl-root junction.

The amount of auxin loaded from the shoot into the root in wild-type seedlings was reduced after 24 h of inoculation with rhizobia, but the auxin was still transferred with the same speed (Fig. 5B). This time point corresponded to the onset of AON (Fig. 1D). The auxin loading and transport in the *sunn* mutant did not change in response to inoculation (Fig. 5D). Previous experiments showed that rhizobia have a local effect on auxin transport and it was therefore suggested that auxin transport regulation was part of the process leading to nodule initiation (Mathesius et al., 1998; Boot et al., 1999). We confirmed these results and showed that both wild type and *sunn* showed a fast and local reduction in auxin transport at the inoculation site (Fig. 6C). Since both wild type and *sunn* form nodules, the idea that this local reduction in auxin transport is associated with nodule initiation is confirmed.

Our results extend our understanding of the role of auxin transport in nodule initiation by showing that auxin transport inhibition by rhizobia is both a local and a long-distance process. Most likely, these two processes are regulated separately, because only the local regulation of auxin transport is effective in *sunn*, whereas the long-distance control is defective. Therefore, we suggest that the long-distance control involves a change in auxin loading following inoculation and is not related to the formation of nodules initiated within the first 24 h after inoculation. Instead, we suggest that this reduction of auxin loading could be part of the autoregulation control. Three arguments support that hypothesis. One is that *sunn*, which lacks AON, did not show a reduction in auxin loading; the second is that the time of AON is correlated with the time of reduction of auxin loading 24 h after inoculation in the wild type; the third argument is that application of NPA below the cotyledons inhibited nodule formation in *sunn* in the zone of the root that is autoregulated in wild type (Fig. 3). In addition, grafting experiments have shown that the autoregulation signal originates in the shoot (Delves et al., 1986; Krusell et al., 2002; Penmetsa et al., 2003).

So far, we do not know how auxin loading could be altered by rhizobia. Rhizobia could either have an effect on the PIN protein(s) or could affect flavonoid synthesis or release, which could alter auxin transport (Brown et al., 2001). Our results from the use of NPA suggest that NPA has an effect on both the speed and the amount of auxin transport in the roots, whereas rhizobia only affected the amount of auxin loaded from the shoot to the root. Therefore, it is possible that rhizobia affect auxin transport or loading via a different mechanism to that of NPA.

Auxin Stimulates Nodule Formation

The most likely involvement of auxin in nodule initiation is its role as a stimulator of cell division (Roudier et al., 2003). In addition to auxin, cytokinins are required for cell division at a certain ratio to auxin (Libbenga et al., 1973). Previously, it had been assumed that nodule initiation in indeterminate legumes is stimulated by a low auxin-to-cytokinin ratio. This argument was supported by the fact that auxin transport inhibitors, which are assumed to lower auxin levels in the root, could trigger pseudonodule initiation (Hirsch et al., 1989), and because studies with an auxin responsive gene (*GH3*) in white clover (*Trifolium repens*) indicate that rhizobia or Nod factors reduce *GH3* expression at the site of inoculation between 12 and 24 h after inoculation (Mathesius et al., 1998). In addition, the application of external auxin stimulates lateral root formation in many species but reduces nodule numbers. In this study, we used direct auxin measurements to quantify auxin levels in whole roots and at the inoculation site (Fig. 7B). We found that the *sunn* mutant, which forms an increased number of nodules, has higher auxin levels in the whole root and shoots than wild-type plants. Therefore, lower auxin levels are not correlated with increased nodulation as previously thought. To underline this, the auxin concentration at the site of inoculation was 3 times higher

in the *sunn* mutant than in the wild type. Our data also suggest that the result of the reduction in auxin loading following inoculation of the wild type with rhizobia is a reduction in auxin levels at the site of inoculation after approximately 24 h. We think that this reduction of auxin levels could be the reason why further nodulation in the root is inhibited after the first nodules have been initiated. In the future, it would be important to determine the exact cellular location of auxin in the wild type and *sunn* mutant during nodulation as small local changes in auxin concentrations could be important determinants of cell division activity.

The increased auxin levels that we measured in *sunn* could also explain the reported phenotypes in *sunn* in the absence of rhizobia. First, root growth and length of cells are reduced in *sunn*, consistent with the inhib-

itory effect of high auxin on root growth. Secondly, Penmetsa et al. (2003) observed that *sunn* root growth is insensitive to external application of ethylene, or its precursor, 1-aminocyclopropane-1-carboxylic acid. This phenotype could be explained by the hypothesis that the inhibition of root growth by ethylene is mediated by ethylene-induced auxin synthesis (Stepanova et al., 2005). If auxin levels are already high in *sunn*, then ethylene may not increase auxin levels further, or the increase in auxin may not inhibit root growth further than it is reduced in *sunn*. This is supported by the finding of Penmetsa et al. (2003) showing that root growth of ethylene-treated wild type is similar to that for ethylene-treated *sunn* plants.

The hypothesis that high auxin levels are necessary to sustain nodulation is in contrast to the auxin burst
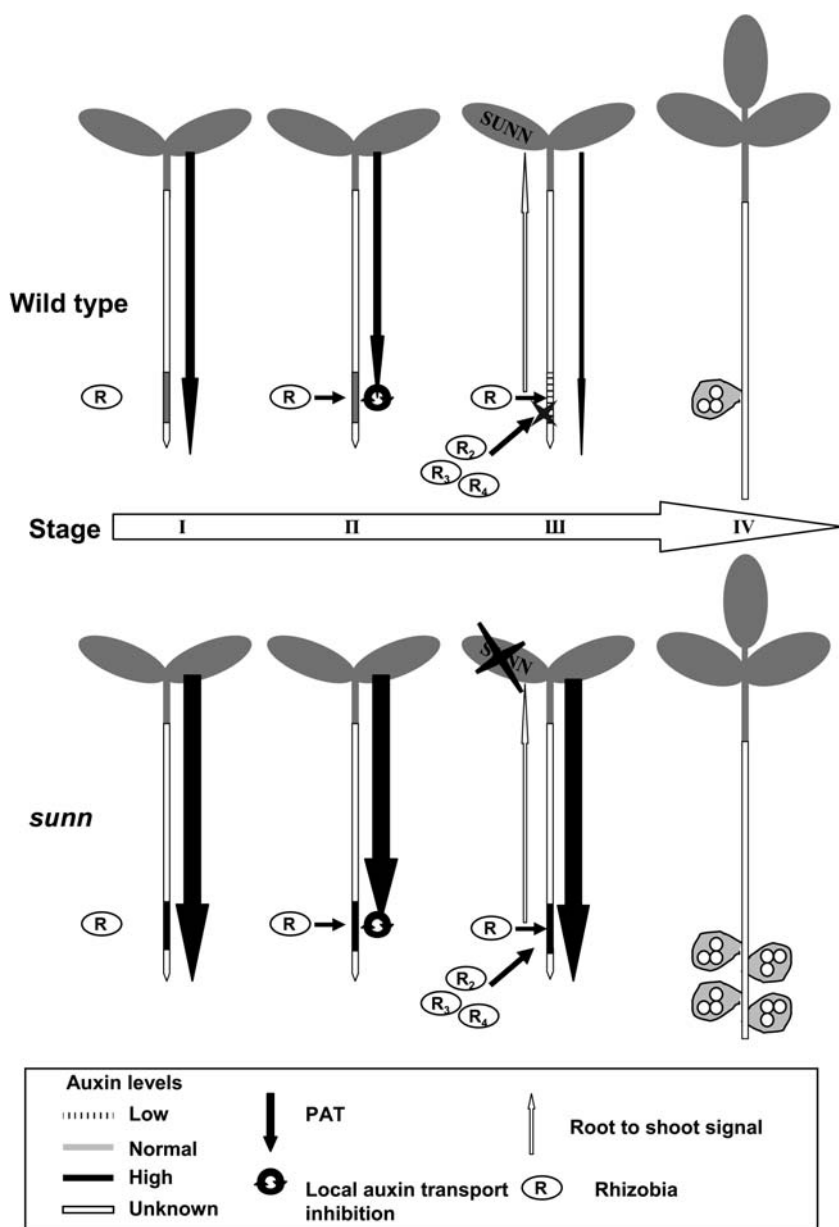
Figure 8. Summary model for the role of auxin in AON in *M. truncatula*. Stage I, Auxin is transported from shoot to the root in both the wild type and the *sunn* mutant at a similar (relative) speed. However, the *sunn* mutant transports more auxin and has higher endogenous auxin levels at the site of inoculation. Stage II, After infection with rhizobia, auxin transport is inhibited at the site of nodule initiation. Stage III, Subsequently, a signal is sent to the shoot from the site of nodule initiation; this is perceived by the LRR-RLK encoded by *SUNN* and leads to a reduction in auxin loading from the shoot to the root. This reduction in auxin (transport) inhibits further nodules from forming in the younger part of the root, because not enough auxin is present in the root to stimulate or sustain nodule formation. In the *sunn* mutant, loading of auxin from the shoot to the root is increased, compared with the wild type, leading to higher auxin levels in the zone of nodule initiation. However, because the LRR-RLK is defective in *sunn* plants, no change in auxin loading from the shoot to the root occurs in response to early nodule formation. Stage IV, The wild type forms limited mature nodules, whereas the *sunn* mutant shows supernodulating roots.

control hypothesis proposed by Gresshoff (1993) for soybean, a determinate legume. Recently, Lohar and VandenBosch (2005) showed that when *L. japonicus* roots were grafted on to *M. truncatula* shoots, rhizobia were able to nodulate the roots, but inoculated *M. truncatula* roots grafted onto *L. japonicus* shoots failed to produce nodules. These findings suggest that determinate and indeterminate legumes require different signaling to initiate nodules. This may include a difference in the hormone levels, as Caba et al. (2000) did not find a difference in auxin levels between the supernodulating soybean *nts* and its wild type, and Pacios Bras et al. (2003) could not find an inhibition in the auxin transport after inoculation in *L. japonicus*. Therefore, there could be some difference in the requirements, transport capacity, or regulation of transport of auxin in determinate and indeterminate legumes.

Summary Model

We hypothesize that a local, transient inhibition of auxin transport at the site of nodule initiation is necessary to initiate nodules. This most likely occurs through blocking auxin efflux from cells near the inoculation site, causing auxin to accumulate where nodules are initiated. Subsequently, the first initiation of nodules sends a so-far-unknown signal to the shoot (Gresshoff, 1993). This signal is perceived directly or indirectly by the LRR-RLK encoded by *SUNN*, or its homologs in other legumes, and leads to a reduction in auxin loading from the shoot to the root. This reduction in auxin (loading) inhibits further nodules from forming in the younger part of the root, because not enough auxin is present in the root to stimulate or sustain nodule formation. In the *sunn* mutant, loading of auxin from the shoot to the root is increased, leading to higher auxin levels in the zone of nodule initiation. However, because the LRR-RLK is defective in *sunn*, no change in auxin loading from the shoot to the root happens in response to early nodule formation. High amounts of auxin continue to be transported to the root and sustain supernodulation. This model is summarized in Figure 8.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Medicago truncatula cv Jemalong genotype A17, which was used as wild-type control, and its derivative *sunn* mutant were scarified with sandpaper, surface sterilized, first, for 10 min with 6.25% (v/v) hypochlorite and washed six times with sterile water. Second, seeds were sterilized with 200 mg L^{-1} Augmentin for 6 h at 29°C, washed six times with sterile water, and germinated on plates containing Fåhreaus (F) medium (Fåhreaus, 1957) containing 0.8% agar (Grade J3, Gelita) in the dark overnight at 29°C. Seedlings were transferred to large petri dishes (150 mL) containing F medium, 10 seedlings per plate. Plates were kept vertical and the bottom one-half of each plate was sealed with Nescofilm and the sides were covered for two-thirds with black paper. An aluminum foil spacer was placed between the lid and the petri dish to allow air exchange. Plates were incubated in a growth chamber at a constant 21°C during a 16-h d and 8-h night with a photon flux density of 100 μmol m^{-2} s^{-1}. Seedlings were flood inoculated with 10 μL of an overnight-grown culture

of *Sinorhizobium meliloti* strain 1021 in Bergensen's modified medium adjusted to an optical density at 600 nm (OD$_{600}$) of 0.1. To study the effect of auxin (transport) inhibitors and (synthetic) auxins on the plant growth and nodulation, PCIB (Sigma Aldrich) and NPA (Casei) were dissolved in methanol, IAA (Sigma Aldrich), NAA (Sigma Aldrich), and 2,4-D (Sigma Aldrich) were dissolved in ethanol, and were added to melted F agar medium just before pouring the medium into the plates. Seedlings were transferred to F medium containing NPA 18 h before inoculation. Plants grown on F medium containing the same concentrations of methanol and ethanol were used as negative control. For the local application of NPA, NPA stock solution (in methanol) was added to a melted agarose solution (1% w/v) to a final concentration of 10^{-5} M. The NPA containing agarose was applied just below the cotyledons with a Pasteur pipette 24 h before inoculation.

To measure cell lengths, 4-d-old roots were stained with 0.1% Methylene Blue for 5 min under vacuum, rinsed in water, and a segment of 1-cm length was excised, starting 2 cm from the root tip. The length of five consecutive cells in the first outer cortical layer was measured with a graticule under a stereomicroscope, using at least 10 seedlings per genotype.

Time Point of AON

To measure the time point of AON, 3-d-old seedlings were inoculated with rhizobia. After inoculation, the root tip was marked with a permanent marker on the back of the petri dish at 0, 24, 48, and 72 h. After 21 d, nodules formed within the root segments corresponding to the different time points were counted.

Long-Distance Auxin Transport Measurements

Auxin transport was measured in 4-d-old seedlings grown on F medium. We applied 12 pmol of [^{3}H]IAA (Amersham Bioscience, specific activity of 850 GBq mmol^{-1}) in 2 μL ethanol between the cotyledons of the seedling. The seedlings were incubated vertically for 3 h and then cut into 5-mm segments (see Fig. 4A) and placed in a scintillation tube with 4.5 mL scintillation fluid (Perkin Elmer). Samples were gently shaken overnight to facilitate extraction before being analyzed in a Beckman LS6500 scintillation counter (Beckman Instruments). To study the effect of an auxin transport inhibitor, NPA was dissolved into methanol and added to melted F agar medium just before pouring the medium into the plates. Seedlings were transferred to F medium containing NPA 18 h before the [^{3}H]IAA was applied.

To determine the extent of metabolism of the transported [^{3}H]IAA, 4-d-old seedlings were supplied with 12 pmol of [^{3}H]IAA and incubated as above. Whole roots of five seedlings were harvested ground in liquid nitrogen and resuspended in 1 mL 80% (v/v) methanol containing 250 mg L^{-1} butylated hydroxytoluene. The suspension was shaken for 18 h at 4°C as described by Beveridge et al. (2000). Extracts were fractionated by reverse phase HPLC (Shimadzu LC-10VP series HPLC) fitted with a diode array UV/VIS detector at 190 to 800 nm, using a C18 column (4.6 × 250 mm; Alltech). Solvents were HPLC grade acetonitrile and deionized water containing 1% (v/v) acetic acid. The solvent program was from 10% (v/v) acetonitrile (hold 2 min) to 80% (v/v) acetonitrile over 25 min linearly at a flow rate of 1 mL/min. The [^{3}H] content of individual 1-min HPLC fractions was determined by scintillation counting.

Local Auxin Transport Measurements

Prior to auxin transport measurements, roots were treated by spot inoculation of rhizobia in the emerging root hairs zone. For spot inoculation, a glass capillary was pulled over a flame, glued to a hypodermic needle, and autoclaved. A small drop of bacteria (OD$_{600}$ = 0.1) was placed at the zone of emerging root hairs under a microscope. For measurements at the hypocotyl-root junction, seedlings were not inoculated. The [^{3}H]IAA solution, 7.5 μL of 1 mCi mL^{-1} (Amersham Biosciences), was diluted in 30 μL of ethanol and mixed into 1.5 mL of melted 4% agarose (approximately pH 4.8) in a small petri dish. Once solid, the agar was cut into 8-mm^3 donor blocks. Each block contained approximately 4 pmol of [^{3}H]IAA. Plant roots were cut 4 mm basipetal from the point of spot inoculation and 4 mm acropetally. The roots or hypocotyl-root segments were then laid on a modified F plate with the basipetal end in contact with a donor block and the acropetal end in contact with a receiver block (empty agar block). The agar blocks were separated from the media by a strip of Parafilm, to prevent diffusion of the [^{3}H]IAA through the agar. The plates were placed vertically in a box and covered with aluminum foil. They were incubated at room temperature for 18 h. The roots were

then cut into two 4-mm segments, above and below the point of treatment (or 4 mm from the hypocotyl-root junction, as judged by the pigmentation of the hypocotyl), and the radioactivity in each segment was analyzed as described above (see Fig. 6A).

Endogenous Auxin Quantification

The roots, shoots, or root segments were frozen and ground in liquid nitrogen. IAA was extracted and their levels quantified using the methods outlined in Jones et al. (2005).

Statistical Analysis

ANOVA, residual maximum likelihood (REML), and Student's *t* tests were calculated using Genstat for Windows (version 8.0, Rothamsted Agricultural Trust). REML was calculated when the data were unbalanced. LSDs were determined at the 0.05 level.

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