The Ethylene-Insensitive *sickle* Mutant of *Medicago truncatula* Shows Altered Auxin Transport Regulation during Nodulation[1][W]

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We studied the ethylene-insensitive, hypernodulating mutant, *sickle* (*skl*), to investigate the interaction of ethylene with auxin transport during root nodulation in *Medicago truncatula*. Grafting experiments demonstrated that hypernodulation in *skl* is root controlled. Long distance transport of auxin from shoot to root was reduced by rhizobia after 24 h in wild type but not in *skl*. Similarly, the ethylene precursor 1-amino cyclopropane-1-carboxylic acid inhibited auxin transport in wild type but not in *skl*. Auxin transport at the nodule initiation zone was significantly reduced by rhizobia after 4 h in both wild type and *skl*. After 24 h, auxin transport significantly increased at the nodule initiation zone in *skl* compared to wild type, accompanied by an increase in the expression of the Mt*PIN1* and Mt*PIN2* (pin formed) auxin efflux transporters. Response assays to different auxins did not show any phenotype that would suggest a defect of auxin uptake in *skl*. The auxin transport inhibitor *N*-1-naphthylphtalamic acid inhibited nodulation in wild type but not *skl*, even though *N*-1-naphthylphtalamic acid still inhibited auxin transport in *skl*. Our results suggest that ethylene signaling modulates auxin transport regulation at certain stages of nodule development, partially through *PIN* gene expression, and that an increase in auxin transport relative to the wild type is correlated with higher nodule numbers. We also discuss the regulation of auxin transport in *skl* in comparison to previously published data on the autoregulation mutant, *super numerary nodules* (van Noorden et al., 2006).

Nitrogen-fixing nodules are formed on the roots of most legumes as a result of symbiotic interactions between a compatible species of soil bacteria generically called rhizobia and its legumes host. The number of nodules formed and the maturation of those nodules are internally regulated by the plant. This process involves positive and negative regulators, which act in concert systemically and locally to control nodule development.

One mechanism controlling nodule numbers is termed autoregulation of nodulation (AON), in which more mature nodules inhibit the formation of nodules in younger root tissues (Caetano-Anollés and Gresshoff, 1991). Grafting studies in these mutants determined that autoregulation is shoot controlled (Carroll et al., 1985; Delves et al., 1988; Jiang and Gresshoff, 2002). It is thought that after inoculation of rhizobia at the root, a signal moves from the root to the shoot where it activates a so far unidentified autoregulation signal that subsequently inhibits new nodules from developing. Mutants defective in AON allow nodules to grow in newly developing roots, resulting in a supernodulation phenotype (Carroll et al., 1985; Sagan and Duc, 1996; Wopereis et al., 2000; Penmetsa et al., 2003). The gene regulating AON has been identified in several legumes and encodes a Leu-rich repeat receptor-like kinase (Krusell et al., 2002; Searle et al., 2003; Schnabel et al., 2005). This mutation can also have various pleiotropic effects on other aspects of plant development as shown in the short root phenotype of the *hypernodulation aberrant root formation* (*har1-1*) mutant of *Lotus japonicus*, the *sym29* mutant of *Pisum sativum* and the *super numerary nodules* (*sunn*) mutant of *Medicago truncatula* (Sagan and Duc, 1996; Wopereis et al., 2000; Schnabel et al., 2005).

In addition, phytohormones are known to control nodule initiation and development (Hirsch, 1992; Hirsch and Fang, 1994; Ferguson and Mathesius, 2003). Auxin appears to be involved at different stages of nodule formation and its main role is thought to be the control of cell division and differentiation as well as vascular bundle formation (de Billy et al., 2001; Roudier et al., 2003). Auxin is transported from the shoot to the root via a polar, active transport system in the xylem parenchyma. Auxin is thought to be imported into cells through auxin import proteins, including AUX1 (Yang et al., 2006) and the P-glycoprotein PGP4 (Terasaka et al., 2005), and exported via auxin

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efflux carrier complexes, including the auxin efflux proteins pin formed (PIN; Petrásek et al., 2006) and multidrug resistance (Geisler et al., 2005; for review, see Benjamins et al., 2005; Blakeslee et al., 2005; Geisler and Murphy, 2006). In addition, it has been suggested that auxin may be transported by phloem transport, because AUX1 is localized to protophloem cells in Arabidopsis (*Arabidopsis thaliana*; Swarup et al., 2001) and may facilitate loading and unloading of auxin into and out of the phloem (Marchant et al., 2002). Studies by Ljung et al. (2001, 2005) showed that treatment of Arabidopsis seedlings with the polar auxin transport inhibitor *N*-1-naphthylphtalamic acid (NPA) does not inhibit all the transport of auxin from the shoot to the root, indicating that auxin may be transported partially by mechanisms not regulated by NPA. However, a direct confirmation of phloem transport of auxin is still missing. Overall, regulation of auxin transport and accumulation has been shown to regulate organ development throughout the plant (Benková et al., 2003), including nodulation.

Within the first few hours after inoculation of roots with rhizobia, a transient inhibition of auxin transport near the site of inoculation has been observed in white clover (*Trifolium repens*; Mathesius et al., 1998) and *Vicia sativa* (Boot et al., 1999). This transient auxin transport inhibition may be necessary for nodulation in indeterminate legumes, and is likely to be mediated by flavonoids (Wasson et al., 2006). Subsequently, auxin accumulates in the inoculation zone before cortical cell divisions in white clover (Mathesius et al., 1998) and bean (*Phaseolus vulgaris*; Fedorova et al., 2000). Auxin is localized in dividing cortical cell, both in white clover (Mathesius et al., 1998) and in *L. japonicus* (Pacios-Bras et al., 2003), as judged by the expression of an auxin responsive promoter (*GH3*) fused to *β-glucuronidase* or *green fluorescent protein. AUX1* (Mt*LAX*) gene expression is localized in nodule primordia in cells close to the vascular bundle of the primary root, suggesting that auxin is channeled into a growing nodule primordium by AUX1 (de Billy et al., 2001). Similarly, Mt*PIN2* is localized to the base of young nodules (Huo et al., 2006). Support for a role of auxin response and transport in nodule initiation comes from studies in *M. truncatula* showing that nodulation is inhibited by the auxin action inhibitor p-chlorophenoxyisobutyric acid (van Noorden et al., 2006) and by silencing of several *PIN* genes by RNA interference (Huo et al., 2006).

Auxin transport is also likely to have a role in controlling nodule numbers. In a recent study of the supernodulating (AON) *M. truncatula sunn* mutant, it was found that the local auxin transport inhibition at the inoculation site occurs in both wild type and *sunn*. However, long distance auxin transport from the shoot to the root was significantly higher in *sunn*. In addition, long distance auxin transport was reduced after inoculation in the wild type, but not in the *sunn* mutant (van Noorden et al., 2006). This suggested that: (1) there are two independent auxin transport systems, (2) that the local auxin transport regulation is necessary for nodule initiation, and (3) that a reduction of long distance auxin transport is correlated with AON. Higher auxin transport from shoot to root, and a higher auxin accumulation at the inoculation site were accompanied by higher nodule numbers in *sunn* (van Noorden et al., 2006).

Ethylene, a gaseous phytohormone, is transiently induced by rhizobia during nodule initiation (Ligero et al., 1986; Caba et al., 1998) and negatively affects the process of nodule development. Ethylene is involved in the inhibition of infection thread growth (Penmetsa and Cook, 1997) and regulates the radial positioning of cortical cell division (Heidstra et al., 1997; Penmetsa et al., 2003). A plant mutant defective in ethylene perception, *sickle* (*skl*), has been identified in *M. truncatula*, which most likely has a mutation in an ortholog of the Arabidopsis *ETHYLENE-INSENSITIVE 2* (*EIN2*) gene, with a single recessive mutation (Penmetsa and Cook, 1997). EIN2 is an early response regulator located in the nuclear membrane, which transmits ethylene signals from the receptor complex to the nucleus, where it activates a number of transcription factors (for review, see Alonso and Stepanova, 2004). The *skl* mutant is insensitive to ethylene in a triple response assay, and likewise, nodulation is not inhibited by ethylene as it is in the wild type. *Skl* shows reduced leaf and petal senescence, increased root length, and a range of nodulation phenotypes, including sustained infection thread growth, hypernodulation, and the occurrence of cell divisions across the radial circumference of the root (Penmetsa and Cook, 1997; Penmetsa et al., 2003). Consistent with the negative role of ethylene on nodulation, the ethylene biosynthesis inhibitor L-α-(2-aminoethoxyvinyl)-Gly and silver ions (Ag^+), which inhibit ethylene sensing, significantly increase nodulation in alfalfa (*Medicago sativa*), soybean (*Glycine max*), *L. japonicus*, and *M. truncatula* (Peters and Chris-Estes, 1989; Lee and LaRue, 1992; Caba et al., 1999; Nukui et al., 2000; Oldroyd et al., 2001). So far, the mechanism of action of ethylene during nodulation is not well understood. Ethylene is likely to act at several stages of nodulation (Guinel and Geil, 2002). One possibility is that ethylene signaling is involved in regulation of defense responses during infection (Penmetsa and Cook, 1997). Another possibility, which is investigated in this study, is that ethylene interacts with auxin transport during nodulation.

The interaction between the two hormones in other aspects of root growth has been demonstrated by genetic and physiological studies. For example, ethylene can inhibit auxin transport (Burg and Burg, 1966; Morgan and Gausman, 1966; Suttle, 1988) and stimulate auxin synthesis (Stepanova and Alonso, 2005), while auxin effects can also be mediated by ethylene. As another example, *ethylene-insensitive root 1* (allelic to *pin2*) shows a defect in gravitropic response and a reduced sensitivity to ethylene (Roman et al., 1995) and the auxin transport inhibitor NPA (Luschnig et al., 1998). These studies in Arabidopsis and studies in pine (Kaska et al., 1999) suggest that some of the effects of

ethylene are via regulation of polar auxin transport and in turn, auxin transport regulation often requires ethylene signaling.

Despite the knowledge of hormone interactions in other aspect of plant development, the interaction of ethylene with auxin transport in nodulation has not been tested so far. Here, we examined the ethylene-insensitive mutant, *skl*, to study the interaction of ethylene signaling with auxin in nodule development of *M. truncatula*. We used techniques recently developed in our lab to measure long distance and local auxin transport during nodulation (van Noorden et al., 2006) to find out how auxin transport regulation in *skl* differs from wild type and from the AON mutant, *sunn*. We also examined possible mechanisms through which ethylene could act on auxin transport in *M. truncatula* by testing whether the *skl* mutation affects the expression of two *PIN* genes encoding auxin efflux proteins and by examining the response of the root to different auxins and NPA. Our results suggest that both long distance and local auxin transport regulation is altered in *skl* during nodulation, and that this defect leads to an increase in auxin transport into the nodulation zone. This increase is similar to that in the *sunn* AON mutant, but the mechanism is root controlled in *skl* and shoot controlled in *sunn*.

RESULTS

The Hypernodulation Phenotype of the *skl* Mutant Is Root Controlled

To determine if the hypernodulation phenotype in *skl* was controlled systemically by the shoot or locally by the root, reciprocal grafts between the wild-type and *skl* plants were made. Upon inoculation with its symbiont, *Sinorhizobium meliloti*, a *skl* shoot grafted onto a wild-type root, showed a wild-type nodulation phenotype (2 ± 1 nodules per plant; Table I). In contrast, when a wild-type shoot was grafted onto a *skl* root, a hypernodulation phenotype was observed, although the average nodule number of grafted *skl* roots was lower than that of the *skl* control graft (15 ± 5 and 24 ± 7, respectively; Table I). Wild type-to-wild type and *skl*-to-*skl* control grafts gave similar results to ungrafted plants. In *L. japonicus*, grafting of a *har1* mutant shoot to a wild-type root resulted in a mutant root phenotype, i.e. short root phenotype (Jiang and Gresshoff, 2002; Krusell et al., 2002). In contrast, we found no significant difference of root length between reciprocal grafts and self-grafted plants of *skl* and wild type (Table I). Together, these results indicate that the hypernodulation phenotype of *skl* is controlled locally by the root tissues; this is in contrast to AON mutants.

Table I. *Nodule numbers and root growth of grafted plants between skl and A17 (wild type)*

Graft (Shoot/Root)	Number of Plants	Nodule Numbers per Plant[a]	Root Growth[a]
			mm
A17/A17	15	3 ± 2 b	44 ± 24 d
A17/*skl*	5	15 ± 5 c	31 ± 13 d
skl/A17	10	2 ± 1 b	32 ± 16 d
skl/*skl*	5	24 ± 7 c	30 ± 13 d

[a]Values are mean ± SD. Values followed by the same lowercase letter in the column are significantly different (Kruskal-Wallis test with Dunn's Multiple Comparison post test; $P < 0.05$).

Long Distance Auxin Transport in the *skl* Mutant

To study the long distance auxin transport in *skl*, we first examined the auxin transport in wild-type and *skl* plants under nonsymbiotic conditions. The auxin movement in 4-d-old seedlings was studied by applying [^{3}H]indole-3-acetic acid (IAA) at the shoot apex between the cotyledons. The amount of radioactivity transported from the shoot apex to the root tip was measured after 3 h of incubation by cutting the root into 5-mm segments and determining the radioactivity in each segment (Fig. 1A). We showed previously that almost all radioactivity is still incorporated in the IAA molecule under these conditions (van Noorden et al., 2006). As shown in Figure 1B, the amount and speed of [^{3}H]IAA transport in untreated *skl* plants was similar to that in wild-type plants.

Because ethylene has been reported to inhibit auxin transport in other plants, we compared long distance auxin transport in *skl* with that in wild type upon treatment with the ethylene precursor 1-amino cyclopropane-1-carboxylic acid (ACC). Treatment of seedlings with 1 μM ACC mixed into the agar plate, for 24 h prior to the transport experiment significantly ($P < 0.05$, ANOVA) reduced auxin transport in wild type (Fig. 1C), but not in *skl* (Fig. 1D). A similar reduction in auxin transport was found in wild-type roots treated with 10 μM ACC ($P < 0.05$, ANOVA; data not shown). When the radioactive counts of all root segments were added up, ACC-treated roots contained approximately 70% of [^{3}H]IAA of control-treated roots (73% in a repeat experiment with 1 μM ACC, 83% in an experiment with 10 μM ACC; Supplemental Fig. 1). To test if auxin transport is inhibited by the known auxin transport inhibitor NPA, seedlings were treated with 1 μM NPA, mixed into the agar plate, for 24 h preceding the auxin transport measurements. As shown in Figure 1E, auxin transport in *skl* plants was significantly ($P < 0.05$, ANOVA) reduced by NPA. This was similar to the response in wild-type plants (data not shown; van Noorden et al., 2006). These results suggest that the long distance auxin transport control in *skl* under nonsymbiotic conditions is similar to wild type, and that ACC, but not NPA, requires ethylene signaling to inhibit auxin transport.

To study the involvement of ethylene in long distance auxin transport during nodulation, the wild-type and *skl* plants were inoculated with *S. meliloti* at the zone of emerging root hairs, the most susceptible zone for nodulation, 24 h before [^{3}H]IAA was applied. This time point was chosen because it showed maximum

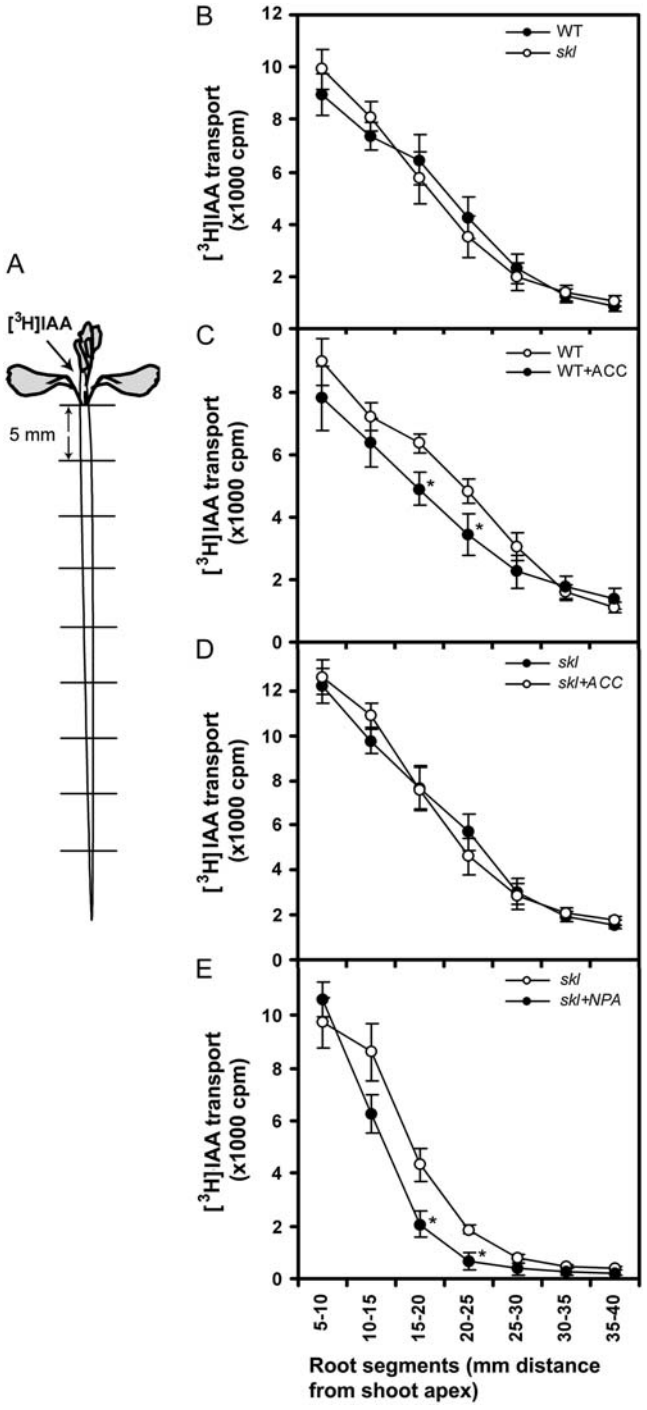

Figure 1. Long distance auxin transport in uninoculated wild-type (WT) and *skl* plants. A, Experimental setup ^{3}H-IAA was applied between the cotyledons of 4-d-old seedlings grown for 24 h on the treatment medium. The tissue below the cotyledons was cut into 5-mm segments, and the radioactivity in each segment was determined in counts per min. B, Auxin transport in wild-type and *skl* plants. C and D, Auxin transport in wild-type (B) and *skl* (C) plants in the presence or absence of 1 μM ACC in the growth medium. E, Auxin transport in *skl* plants in the presence and absence of 1 μM NPA in the growth medium. Values are means ± SE, n = 16. Two-way ANOVA was calculated for treatment and position effect, and if $P < 0.05$, post tests were carried out for pairwise comparisons. Stars indicate segments that are significantly different from their corresponding control (Student's t test, $P < 0.05$).

long distance auxin transport inhibition in *M. truncatula* after inoculation in similar assays before (van Noorden et al., 2006). This time point also correlated with the onset of ethylene evolution following Rhizobium inoculation in Medicago and soybean roots (Ligero et al., 1986; Caba et al., 1998). Inoculation significantly ($P < 0.05$) reduced [^{3}H]IAA transport into wild-type roots (Fig. 2A). This inhibition was slightly but not significantly further ($P > 0.05$) reduced by simultaneous treatment with 1 μM ACC (Fig. 2A). In contrast to wild type, *S. meliloti* inoculation had no significant effect on [^{3}H]IAA transport in *skl* (Fig. 2B). Likewise, treatment of inoculated *skl* plants with ACC did not reduce [^{3}H]IAA transport (Fig. 2B). However, treatment of *skl* roots with NPA for 24 h prior to inoculation with rhizobia caused a similar reduction of auxin transport as with NPA alone (data not shown). These results demonstrate that the *skl* mutation abolishes the inhibition of long distance auxin transport by rhizobia.

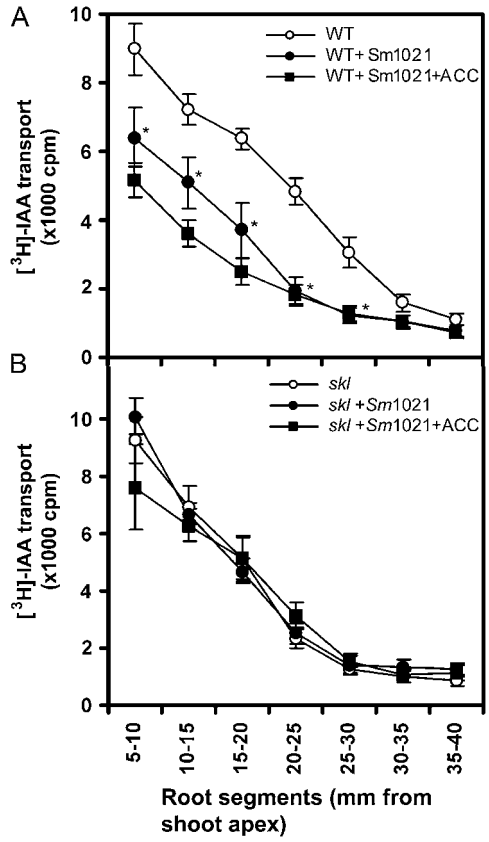

Figure 2. Long distance auxin transport in inoculated wild-type and *skl* plants. ^{3}H-IAA was applied between cotyledons of 4-d-old seedlings 24 h after inoculation (and optional ACC treatment). The tissues below cotyledons were cut into 5-mm segments, and the radioactivity of each segment was determined in counts per min as shown in Figure 1A. A, Auxin transport in wild type inoculated with *S. meliloti* at the zone of emerging root hairs (approximately 3–5 mm from the root tip) and treated with 1 μM ACC. B, Auxin transport in *skl* inoculated with *S. meliloti* and treated with 1 μM ACC (±SE, n = 16). Stars indicate segments that are significantly different from their corresponding control (Student's t test, $P < 0.01$).

Local Auxin Transport in the Nodulation Zone of *skl* Plants

Previous studies have shown that local regulation of auxin transport at the inoculation site is independent of long distance auxin transport control and that it might be necessary for nodule initiation (van Noorden et al., 2006; Wasson et al., 2006). We therefore hypothesized that local auxin transport regulation by rhizobia would be observable in *skl*. To test local auxin transport, an agar block ($2 \times 2 \times 4$ mm^3) containing a test compound, or a small drop of rhizobia, was applied on the emerging root hair zone of 3-d-old seedlings. After 4 or 24 h incubation, the roots were cut, and a donor agar block containing [^{3}H]IAA was placed on the basal end of the root segment 6 mm above the center of the treatment application (Fig. 3A). After 6 h of transport, the [^{3}H]IAA content was determined in two segments of the root, excised 4 mm above the center of the treatment application (top segment), and 4 mm below the center of the treatment application (bottom segment). This experiment can determine the total auxin uptake from the donor block, as well as auxin transport from the top to the bottom segment.

By reversing the position of the [^{3}H]IAA donor block to the apical, rather than the basal end of the root segment in this region of the root, we determined that acropetal transport (from the basally placed auxin donor block toward the root tip) was more than 10 times higher than basipetal transport (from the apically placed block toward a segment 1 cm from the root tip). Approximately 16% of total applied [^{3}H]IAA was transported to the root segment in acropetal direction and only 1.2% in basipetal direction ($P < 0.05$; Student's t test, $n = 10$; data not shown). All experiments described below are for acropetal auxin transport (Fig. 3A).

As shown in Figure 3B, the [^{3}H]IAA content in the top root segments was more than 2-fold higher in control wild-type than in control *skl* roots, whereas [^{3}H]IAA level was similar in the bottom segment of control wild-type and *skl* roots. This increased [^{3}H]IAA level could point to decreased uptake or loading of auxin from the donor block into the root in *skl*. ACC treatment had no effect on [^{3}H]IAA transport into the top segment of wild-type or *skl* roots. In contrast, ACC treatment significantly ($P < 0.05$) reduced the auxin transport into the bottom segment of wild-type roots, acropetal from where the ACC was placed. No reduction of auxin transport was observed in *skl* roots after ACC treatment. Thus, similar to the results found in the long distance auxin transport experiment, local auxin transport inhibition by ACC requires ethylene signaling through SKL.

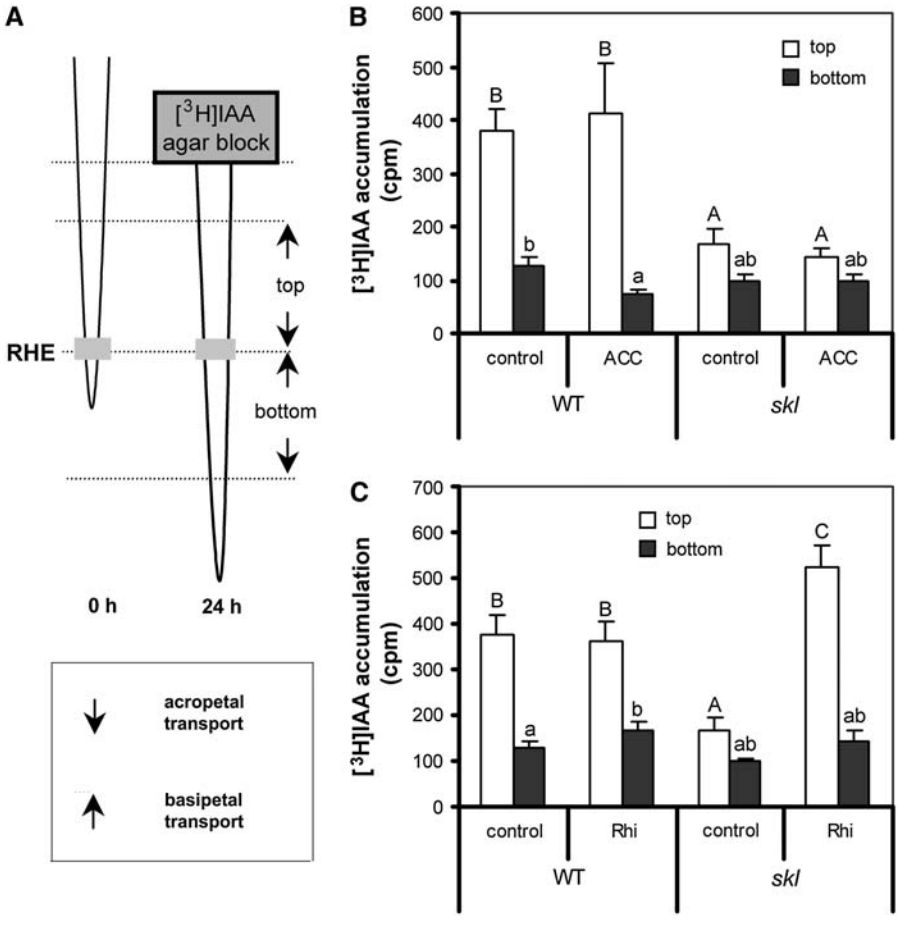

Figure 3. Local auxin transport in wild-type and *skl* plants after treatment with 1 μM ACC or Rhizobium inoculation. A, Experimental setup. An ACC-containing agar block or a drop of *S. meliloti* was positioned at the root hair emergence zone (RHE, gray box). [^{3}H]IAA-containing agar blocks were placed on the basal end of root segments 24 h later. After 6 h of transport, the radioactivity of the root segment within 4 mm above (top) and below (bottom) the site of ACC application or Rhizobium inoculation was determined in counts per min. B, [^{3}H]IAA content in ACC-treated plants. C, [^{3}H]IAA content in control and inoculated (Rhi) plants. Values are mean ± SE, $n = 20$. Statistical analysis was done separately for the top and bottom segment of the roots. Bars with different uppercase or lowercase letters (for top and bottom segments, respectively) are significantly different at the $P < 0.05$ level (one-way ANOVA).

To examine the local auxin transport in the nodulation zone, we spot inoculated the root at the zone of emerging root hairs with *S. meliloti*, applied the agar block containing [^{3}H]IAA 6 mm above the inoculation site 4 or 24 h later, and then measured the [^{3}H]IAA transport as described above. After 4 h, reduced auxin transport was measured in wild-type and *skl* roots into the bottom segment of the root after inoculation ($P < 0.05$, $n = 12$–20). The amount of auxin was reduced by 23% and 43%, respectively, in wild type and *skl* (data not shown). After 24 h, *S. meliloti* inoculation increased [^{3}H]IAA accumulation in the bottom segments in the wild type (Fig. 3C). In marked contrast, *S. meliloti* inoculation increased [^{3}H]IAA accumulation in the top segment of the *skl* roots to 3-fold compared to controls, but had no significant effect on [^{3}H]IAA accumulation in the bottom segment (Fig. 3C). These results suggest that the *skl* mutation (1) does not abolish the early transient local auxin transport inhibition but (2) subsequently increases the local auxin transport or uptake into the root and causes auxin accumulation of acropetally transported auxin in or just above the nodulation zone shortly before nodule initiation.

Possible Mechanisms of Auxin Transport Regulation by Ethylene Signaling

To examine possible mechanisms by which ethylene affects auxin transport during nodulation, we examined if the expression levels of Mt*PIN1* and Mt*PIN2* in *skl* plants were altered by *S. meliloti* inoculation. Root segments representing the nodulation zone (1–1.5 cm length) of inoculated plants were harvested 24 h after inoculation. In parallel to this, equivalent root segments of mock-inoculated plants were harvested as controls. In the absence of *S. meliloti*, the expression levels of Mt*PIN1* and Mt*PIN2* mRNA were similar ($P > 0.05$) between wild type and *skl* (Fig. 4, A and B). When inoculated with *S. meliloti*, the Mt*PIN1* mRNA level of wild type was similar ($P > 0.05$) to that of mock-inoculated control (Fig. 4A), whereas the Mt*PIN2* expression was slightly but significantly ($P < 0.05$; Student's *t* test) increased (Fig. 4B). In *skl*, both Mt*PIN1* and Mt*PIN2* expression levels were significantly increased following inoculation with *S. meliloti* ($P < 0.05$; Fig. 4, A and B). These results support our observations of increased auxin transport in *skl* in the nodulation zone (compare with Fig. 3C).

As *PIN* gene expression levels did not explain the reduction in auxin uptake in untreated *skl* roots, we treated plants with different auxins that have different requirements for auxin uptake and export through AUX1 and PIN proteins. To compare the sensitivity to auxin in wild type with *skl* we treated roots with concentrations of 0.1 to 10 μM IAA, naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and indole butyric acid (IBA) and measured root growth and nodulation. The plants were grown on medium containing auxin from 24 h before inoculation with *S. meliloti*. The positions of root tips at the time of inoculation were marked on the plates and measured again 2 weeks later. IAA inhibited the root elongation of wild-type and *skl* plants, and similar effects were obtained with 2,4-D and NAA treatment. However, *skl* roots showed a greater resistance toward the inhibitory effect of either IAA, 2,4-D, or NAA than wild type, and *skl* roots were insensitive to the inhibitory effect of IBA (Supplemental Fig. 2).

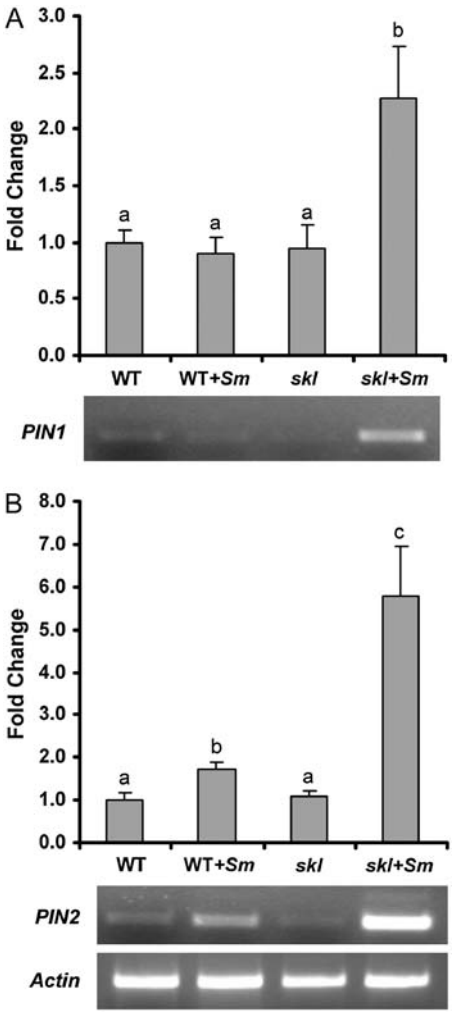

Figure 4. Expression analysis of Mt*PIN1* and Mt*PIN2* in wild-type A17 (WT) and *skl* roots with or without Rhizobium inoculation. Three-d-old seedlings were inoculated with *S. meliloti*, and the root segments representing the nodulation region (without root tip) were harvested 24 h later and used to extract total RNA. mRNA levels were determined by RT-PCR analysis. Actin was used as a control. Values are mean ± SE from three independent experiments with approximately 20 roots each. Values are normalized to controls, and shown as relative fold change to uninoculated wild type. Bars with different letters are significantly different at the $P < 0.05$ level (one-way ANOVA).

Exogenous auxins also inhibited nodulation in wild-type and *skl* plants. In contrast to the greater resistance of *skl* to inhibition of root growth by auxin, the extent of IAA-induced inhibition of nodulation in *skl* was similar to that found in wild type ($P > 0.05$), in which 1 μM IAA almost completely inhibited nodulation

(Fig. 5A). NAA and 2,4-D treatments also reduced nodulation of *skl* and wild type in a similar fashion to IAA, with 1 μM almost completely inhibiting nodulation in both genotypes (data not shown). In contrast, 1 μM IBA had no effect on nodule numbers in wild type, but it significantly reduced nodule numbers in *skl* (Fig. 5B). However, the total number of nodules in *skl* was still higher than in wild type at $\leq$1 μM IBA.

To test the effect of reduced polar auxin transport on root growth and nodulation in wild type and *skl*, roots were treated with the auxin transport inhibitor NPA from 24 h before inoculation. As shown in Figure 6, A and B, NPA reduced root elongation and nodulation of wild type to 40% and 50%, respectively. In contrast, nodulation and root growth of *skl* were not reduced by NPA.

DISCUSSION

Our study aimed at answering three questions about the involvement of ethylene during nodulation: (1) Does ethylene affect the long distance transport of auxin from shoot to root, (2) does ethylene affect local auxin transport at the inoculation site, and (3) what is the possible mechanism for the action of ethylene on auxin transport during nodulation? In addition, we compared auxin transport regulation in *skl* with that to the autoregulation (AON) mutant *sunn* in *M. truncatula*.

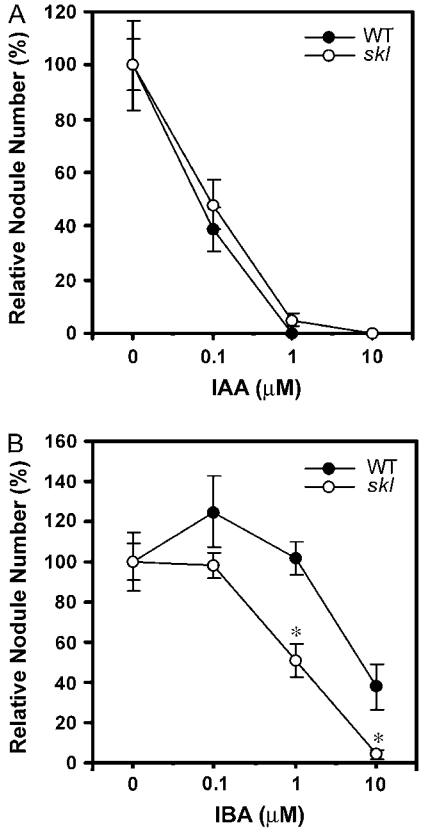

Figure 5. Nodulation responses of wild-type and *skl* plants to IAA and IBA. A, Relative nodule numbers in response to IAA. The average total nodule numbers in the control wild-type and *skl* plants were 3.2 and 28.1, respectively. B, Relative nodule numbers in response to IBA. All values are means $\pm$ SE, $n = 10$ to 15. Stars indicate treatments that are significantly different from their corresponding wild-type treatment (one-way ANOVA with Tukey-Kramer multiple comparison test, $P < 0.05$). The average total nodule numbers in control wild-type and *skl* plants were 3.0 and 26.6, respectively.

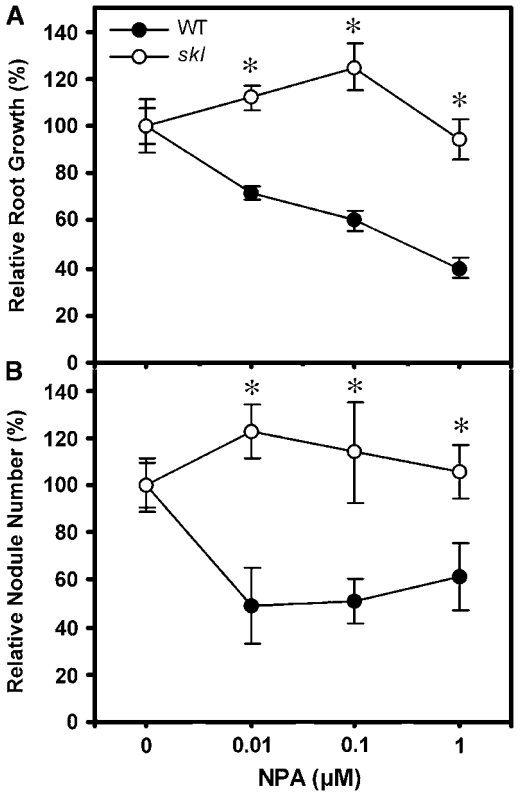

Figure 6. Root and nodulation responses of inoculated wild-type (WT) and *skl* plants to NPA. A, Relative root length in response to NPA. B, Relative nodule numbers in response to NPA. All values are means $\pm$ SE, $n = 15$. Stars indicate treatments that are significantly different from their corresponding wild-type treatment (one-way ANOVA with Tukey-Kramer multiple comparison test, $P < 0.01$). The average total root lengths in control wild type and *skl* were 49 and 40 mm, respectively, and the average total nodule numbers in control wild-type and *skl* plants were 3.8 and 24, respectively.

Effect of Ethylene Signaling on Long Distance Auxin Transport Regulation

Inoculation of wild-type roots with rhizobia at the zone of emerging root hairs led to a significant reduction in auxin transport from the shoot to the root, consistent with previous results in *M. truncatula* (van Noorden et al., 2006). A similar reduction in auxin transport could be achieved by application of the ethylene precursor ACC to the growth medium. This agrees with previous reports on the action of ethylene as an auxin transport inhibitor in various nonlegumes (Burg and Burg, 1966; Morgan and Gausman, 1966; Suttle, 1988). Auxin transport measurements in *skl*

showed that ethylene signaling is required to mediate the inhibition of auxin transport from the shoot to the root by both ACC and by rhizobia. As a result, auxin transfer to the root was not decreased in inoculated *skl* compared to inoculated wild-type roots. This was correlated with higher nodule numbers in *skl*. Similarly, increased auxin transport from shoot to root in the AON mutant *sunn* was also correlated with higher nodule numbers (van Noorden et al., 2006). We therefore hypothesize that the amount of auxin transferred from the shoot to the root might be one determinant of total nodule numbers in *M. truncatula*.

Long distance auxin transport from the shoot to the root in untreated *skl* plants was similar to the wild type, and different responses were only observed after ACC or Rhizobium inoculation. Both ACC and rhizobia stimulate internal ethylene evolution inside the roots (Ligero et al., 1986; Caba et al., 1998). It appears, therefore, that long distance auxin transport is only affected once internal ethylene concentrations change.

Effect of Ethylene Signaling on Local Auxin Transport Regulation

Previous studies have shown that inoculation of roots with rhizobia leads to a transient inhibition followed by an accumulation of auxin around the inoculation site and then in dividing cortical cells (Mathesius et al., 1998). Our results here have confirmed that Rhizobium inoculation resulted in auxin transport inhibition after 4 h, followed by an increase in the uptake of [^{3}H]IAA in the root region just below the inoculation site (bottom segments) of the wild-type roots at 24 h post inoculation, which precedes the first cell divisions in *M. truncatula*. In *skl*, a transient inhibition of auxin transport after 4 h occurred, indicating that this inhibition is not ethylene dependent. Instead, this step is likely to be regulated by flavonoids (Wasson et al., 2006) that might act on *PIN* gene expression (Peer et al., 2004). The early inhibition of auxin transport was followed by an exaggerated increase in IAA accumulation in the top segments of roots, the root region above the inoculation site. This was similar to the *sunn* mutant, where auxin content at the inoculation site is approximately three times higher than in the wild type (van Noorden et al., 2006). These results are supported by the increased expression of the auxin responsive gene *GH3* in both *skl* and *sunn* during early stages of nodulation (Penmetsa et al., 2003). Auxin could act positively inside the root both to promote cell divisions in nodule primordia (Roudier et al., 2003) and to direct nodule organogenesis, as has been demonstrated for other plant organs (Benková et al., 2003). Intriguingly, nodulation is inhibited by high levels of external auxin (Fig. 5), but stimulated by very low auxin concentrations (van Noorden et al., 2006). Most likely, auxin concentration, location, and response in target cells have to be within a narrow window, and this requirement might change over time during the nodule development program.

Apart from the effect of *skl* on auxin transport after inoculation, *skl* also had a significant negative effect on the amount of auxin taken up into the top root segment in the absence of rhizobia (Fig. 3, B and C). This was surprising because ethylene inhibits auxin transport and therefore we expected that lack of ethylene signaling increased auxin transport. Auxin transport is also affected by auxin itself (Peer et al., 2004) and by flavonoids (Murphy et al., 2000; Brown et al., 2001; Buer and Muday, 2004; Wasson et al., 2006). It is possible that the reduced auxin transport capacity in untreated *skl* roots is caused by feedback regulation between auxin, ethylene, and flavonoids, and it would be interesting to test whether flavonoid content is changed in *skl*.

Possible Mechanisms of the Action of Ethylene in Auxin Transport Regulation during Nodulation

Both the long distance and local auxin transport assays confirmed that ACC inhibits auxin transport in *M. truncatula* and requires signaling through SKL. To test whether ethylene might act via regulation of transcription of auxin transport proteins, we measured Mt*PIN1* and Mt*PIN2* expression. Mt*PIN2* is the most likely ortholog of At*PIN2* (Schnabel and Frugoli, 2004). Mt*PIN2* expression was increased after inoculation in the wild type. In *M. truncatula*, Mt*PIN2* is root specific, expressed in early nodule primordia, and its silencing by RNA interference significantly reduced nodule numbers (Huo et al., 2006). The increase in Mt*PIN2* expression after inoculation therefore supports its role in early nodule development. In *skl* an exaggerated increase in both Mt*PIN1* and Mt*PIN2* was found after inoculation. These results may explain the increase of [^{3}H]IAA accumulation in the nodulation zone of *skl* roots 24 h after inoculation.

To determine indirectly whether ethylene signaling is required for auxin uptake as well as export, and to examine whether ethylene mediates similar responses during nodulation as during root growth, we treated plants with different auxins and auxin transport inhibitors. Influx of IAA and 2,4-D into cells is mediated by auxin-influx transmembrane proteins such as AUX1, while uptake of NAA occurs readily by membrane diffusion (Delbarre et al., 1996). Therefore, the *aux1* mutant is more resistant to IAA and 2,4-D, but shows normal sensitivity toward NAA on root growth (Pickett et al., 1990; Marchant et al., 1999; Rahman et al., 2001). *Skl* root growth showed similarly reduced sensitivity to the inhibitory effect of IAA and 2,4-D as to NAA. Therefore, *skl* does not appear to affect auxin uptake via *AUX1*. The auxin IBA was shown to be transported independently of either AUX1 or PIN proteins in Arabidopsis (Rashotte et al., 2003). *Skl* root growth was insensitive to application of IBA, consistent with the characteristics of other ethylene-insensitive mutants, for example *ein2-1* and *etr1-1* mutants of Arabidopsis, and *never ripe* of tomato (*Lycopersicon esculentum*; Clark et al., 1997; Zolman et al., 2000). Root

growth in *skl* was also insensitive to NPA, similar to the response in the Arabidopsis *ein2* mutants (Fujita and Syono, 1996), confirming that NPA requires ethylene signaling to reduce root growth in *M. truncatula*.

Our results further suggest that ethylene plays a different role in mediating auxin responses during root growth and nodulation. Whereas root growth in *skl* was less sensitive to IAA, NAA, and 2,4-D than the wild type, nodulation was similarly inhibited in *skl* and wild type by all three auxins (Fig. 5), suggesting that these auxins do not require ethylene signaling for inhibition of nodulation. In contrast, nodulation in *skl* was more sensitive to IBA inhibition than the wild type, even though the primary root growth appeared normal after IBA treatment. Therefore, the role of ethylene seems to differ between auxins that require AUX1 and/or PIN transport and those that do not.

The response to NPA in *skl* showed similar insensitivity for nodulation as well as root growth, suggesting that in both cases NPA acts via ethylene signaling. This is consistent with findings by Suttle (1988) who showed that ethylene does not directly compete with NPA for sites on its binding protein(s). However, the mechanism by which NPA acts is not clear because NPA still reduced auxin transport in *skl* (Fig. 1E). This could first suggest that nodule numbers in *skl* are not influenced as much by the amount of auxin transport from the shoot as for example in the *sunn* mutant, where NPA reduced nodule numbers significantly (van Noorden et al., 2006). Alternatively, the action of NPA on nodulation may not be restricted to its action on auxin transport itself. Instead, its negative effect might be offset by changes in internal cellular auxin concentration and altered auxin response in *skl*, as application of NPA has been shown to increase the auxin response in the root tip (Sabatini et al., 1999). Ljung et al. (2005) showed that NPA increases auxin levels in root tips of young Arabidopsis seedlings, but that within a few days the root becomes increasingly independent of auxin transport from the shoot as it synthesizes auxin in root tissues. Future experiments could test whether NPA-treated *skl* roots have altered auxin synthesis or response in the root that could affect nodule formation independently of NPA-regulated polar auxin transport. In addition, other auxins than IAA, for example IBA, could be involved in nodulation, and their transport role will have to be determined.

Comparison of Auxin Transport in the *skl* and *sunn* Mutants

Both the *skl* and *sunn* mutants of *M. truncatula* show increased nodule numbers. However, nodule numbers in *sunn* are increased through a Leu-rich repeat receptor kinase acting in the shoot as part of the AON mechanism (Schnabel et al., 2005), whereas *skl* regulates

Table II. *Summary of root and nodule phenotypes of skl and sunn mutants*

The table shows the phenotypes of each mutant compared to wild-type plants. Phenotypes indicated in bold are different between *skl* and *sunn*. ND, Not determined; pi, post inoculation; WT, wild type.

Phenotype	*Skl* Compared to WT	*Sunn* Compared to WT
Uninoculated roots		
Root length	**Longer**[a]	**Shorter**[b,c]
Long distance auxin transport	**Similar**[a]	**Increased**[b]
Local auxin transport	**Lower**[a]	**Similar**[b]
PIN1 and *2* expression	Similar[a]	Similar[d]
Auxin transport inhibition by NPA	Similar[a]	Similar[b]
Auxin transport inhibition by ACC	Less sensitive (insensitive)[a]	ND
Inhibition of root growth by ethylene	Less sensitive[e]	Less sensitive[c]
S. meliloti inoculated roots		
Nodule numbers	Higher[a]	Higher[b]
Nodule distribution	**First inoculation zone**[a,e]	**Along whole root**[b,c]
Nodule position (radial)	**Across entire root**[c,e]	**Opposite xylem poles only**[c]
Nodulation phenotype controlled by	**Root**[a]	**Shoot**[c]
Long distance auxin transport (24 h pi)	Less (not) inhibited by rhizobia[a]	Less (not) inhibited by rhizobia[b]
Local auxin transport (4 h pi)	Similar[a]	Similar[b]
Local auxin transport (24 h pi)	**Higher**[a]	**Similar**[b]
PIN 1 and *2* expression (24 h pi, inoculation zone)	**Higher**[a]	**Similar**[d]
GH3 expression (4–84 pi)	Higher[c]	Higher[c]
Root growth inhibition by auxin	**Less sensitive**[a]	**Similar**[b]
Inhibition of nodulation by auxin	Similar (IAA, NAA, 2,4D), but more sensitive (IBA)[a]	Similar (IAA, NAA, 2,4D; ND for IBA)[b]
Root growth inhibition by NPA	**Less sensitive**[a]	**Similar**[b]
Inhibition of nodulation by NPA	**Less sensitive**[a]	**More sensitive**[b]
Inhibition of nodulation by ethylene	**Less sensitive**[e]	**Similar**[c]

[a]This study. [b]van Noorden et al. (2006). [c]Penmetsa et al. (2003). [d]G. van Noorden, personal communication. [e]Penmetsa and Cook (1997).

nodule numbers through an ethylene response regulator acting in the root (Table II). The transfer of auxin from the shoot to the root was previously found to be increased in the AON mutant *sunn* (van Noorden et al., 2006). It was suggested that long distance auxin transport inhibition from shoot to root by rhizobia might either be the AON signal or might be strongly correlated with AON because (1) auxin transport inhibition coincided with the onset of AON, (2) auxin transport was not inhibited by rhizobia in *sunn*, and (3) NPA application at the shoot-to-root junction significantly reduced nodule numbers in *sunn*. Our results from the *skl* mutant show that auxin transport inhibition might not be unique to AON. First, our grafting experiments between *skl* and wild-type plants have demonstrated the role of roots in the hypernodulation phenotype in *skl* (Table I). Moreover, addition of the ethylene synthesis inhibitor L-α-(2-aminoethoxyvinyl)-Gly to *V. sativa* roots in a split root system does not abolish AON (van Brussel et al., 2002) and *sunn* and *skl* act in separate regulatory pathways (Penmetsa et al., 2003). Therefore ethylene signaling or synthesis is unlikely to affect the AON signal. Our long distance auxin transport experiments showed that rhizobia are equally unable to reduce auxin transport from shoot to root in *skl* as in *sunn* at the time that the AON signal acts in the wild type (Table II), suggesting that long distance auxin transport inhibition can be regulated independently by the shoot and the root (van Noorden et al., 2006).

In uninoculated roots, auxin transport from shoot to root was approximately 3 times higher in *sunn* than in wild type, but similar in *skl* compared to wild type (Table II). In contrast, local auxin transport at the inoculation zone was similar in *sunn* and wild type, but reduced in *skl*, even though in both mutants, Mt*PIN1* and *2* expression was similar to wild type in that root region (Table II). These results, together with the different sensitivities of *skl* and *sunn* to auxins and NPA (Table II), suggest that *sunn* and *skl* act on the auxin transport machinery by two different mechanisms. Future experiments will be directed at characterizing the mechanisms through which *SKL* and *SUNN* act on auxin transport.

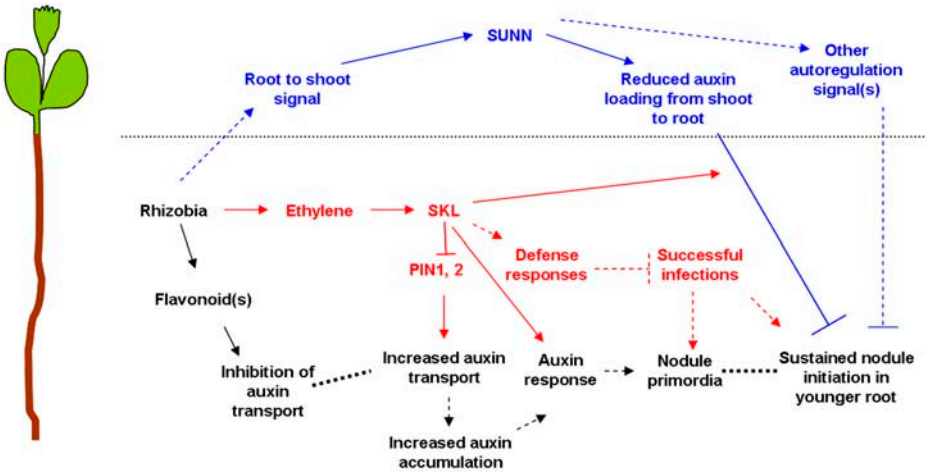

Figure 7. Working model for the involvement of ethylene in nodule formation. The model shows the proposed influence of ethylene on auxin transport in relation to other factors that were shown to affect auxin transport and accumulation. Arrows indicate an effect of one to a subsequent step. Blunt-end lines indicate inhibition. Solid lines show relationships for which evidence has been shown, although each arrow is likely to consist of several steps. Dashed lines show proposed relationships for which no firm evidence exists. A bold dotted line without arrowhead indicates followed by, without a necessary functional relationship. A fine dotted line at the junction between the shoot and the root indicates processes regulated by or taking place in the shoot or root. Black, positive regulation of nodule initiation: We hypothesize that part of the mechanism to initiate a nodule is the regulation of auxin transport via flavonoids, which cause a transient inhibition of polar auxin transport. This is followed by an increase in auxin transport at the nodule initiation site. Auxin accumulates at the nodule initiation site, although it is not clear if this is a result of auxin transport change or if it is regulated independently by rhizobia. If auxin is within a certain concentration window, it might stimulate auxin responses in the inner cortex and pericycle that lead to nodule initiation. Blue, involvement of autoregulation: Autoregulation inhibits sustained nodule initiation in the younger parts of the root system via a root-to-shoot signal (unidentified) which (in) directly activates the SUNN receptor-like kinase (the origin of this signal is not known; most likely it originates not from rhizobia but from a step during early nodule initiation). This leads to a reduction in auxin loading in the shoot that might limit nodule development. In parallel, other so far unidentified autoregulation signals could be active to inhibit further nodulation. Red, the inhibitory actions of ethylene on auxin transport and defense responses. Ethylene is induced by rhizobia and partially inhibits nodulation by mediating defense responses that limit infections and successful nodules (Penmetsa et al., 2003). In addition, our data suggest that ethylene modulates auxin transport and responses in the root. One mechanism is a local decrease in auxin accumulation in the nodulation zone, partially by changing *PIN* gene expression, while ethylene signaling in not necessary for the initial auxin transport inhibition (by flavonoids). SKL is also required to inhibit long distance auxin transport along the root in response to rhizobia.

CONCLUSION

Our data support a model in which local auxin transport inhibition is necessary to initiate nodulation, but this does not require ethylene signaling through SKL. This is followed by increased local auxin transport into the nodule initiation zone, whereby ethylene signaling negatively affects auxin uptake into the nodulation zone, partially by affecting *PIN1* and *2* gene expression. The regulation of nodule numbers and of long distance auxin transport by *skl* is different from that in the AON mutant *sunn*, as *SKL* acts in the root and *SUNN* acts in the shoot. Overall, both hypernodulation mutants show increased auxin transport into the primary nodulation zone by local and long distance auxin transport regulation. We hypothesize that this increase in auxin transport into the primary nodulation site is necessary to sustain higher nodule numbers, which are induced by higher numbers of successful infection events in *skl*. A model summarizing our data is shown in Figure 7.

MATERIALS AND METHODS

Plant and Bacterial Growth Conditions

Seeds of the *Medicago truncatula skl* mutant were obtained from D.R. Cook (Penmetsa and Cook, 1997). Seeds of cv Jemalong A17 were used as the wild type. Seeds were scarified and surface sterilized with 6.25% (v/v) sodium hypochlorite for 15 min. After six washes with sterile water, seeds were spread on nitrogen-free Fåhraeus (F) medium (Fåhraeus, 1957) containing 0.8% agar (grade J3, Gelita). A drop of sterile water was applied on each seed to prevent the seeds from drying. Seeds were incubated at 4°C for 2 d to break their dormancy. Seeds were then germinated by incubating in the dark at 28°C overnight. Seedlings with similar root length were selected and transferred to 15-cm petri dishes containing F medium. The seedlings were incubated vertically in a growth chamber with a light intensity of 90 μmol m^{-2} s^{-1}, and 16 h of light per day at 20°C for 2 d. To reduce light intensity around roots, pieces of dark paper were placed over the lower half of the plates. Two days later, the seedlings were transferred to fresh F medium plates containing any hormone treatment, and incubated in the same growth chamber. After 24 h, the seedlings were inoculated with 5 μL of diluted bacterial suspension. *Sinorhizobium 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 (OD_{600}) of 0.1 to 0.2 or approximately 10^7 cells mL^{-1} (as tested by plate counts). As controls, roots were inoculated with an equivalent amount of diluted Bergensen's modified medium.

Grafting Experiments

Seeds were surface sterilized, germinated overnight at 28°C in the dark, and grown on 15-cm petri dishes containing F medium. The seedlings were incubated vertically in a growth chamber with a light intensity of 90 μmol m^{-2} s^{-1}, and 16 h of light per day at 20°C for 3 d. The grafting technique essentially followed Penmetsa et al. (2003) with the following modifications: To prepare plant material for grafting, 3-d-old seedlings with similar hypocotyl diameters were selected. Seedlings of *skl* and wild-type plants were cut transversely within the chlorophyll-containing hypocotyl region using a fresh razor blade under aseptic conditions. For rootstocks, the hypocotyls were further cut longitudinally for 3 mm. For scions, the hypocotyls were cut to form a v-shape end. The cut end of a scion was then inserted between the cut end of a rootstock, and the graft junction sealed with a piece of Nescofilm that was carefully wrapped around the graft junction. Grafted plants were transferred on F medium plates containing 0.1 mM KNO_3 and allowed to recover on the agar plates by incubating in a vertical position in a growth chamber for 7 d at a light intensity of 90 μmol m^{-2} s^{-1}, and 16 h of light per day at 20°C. Successful grafts, indicated by fresh root growth, were then inoculated with *S. meliloti* as described above.

Long Distance Auxin Transport Assays

Auxin transport measurements were conducted as described in van Noorden et al. (2006). Seeds were germinated, transferred to the treatment medium, and inoculated as described above. After inoculation with *S. meliloti*, the seedlings were incubated in the growth chamber for another 24 h. Subsequently, 2 μL of 8.7 μM [^{3}H]IAA (Amersham Bioscience; specific activity of 850 GBq $mmol^{-1}$) in ethanol were applied directly between the cotyledons (Fig. 2A). The seedlings were then incubated vertically in a growth chamber for 3 h. The plant region below cotyledon was sectioned into 5-mm long segments, and each segment placed into 4 mL of scintillation fluid (Perkin Elmer) overnight while shaking. The segment closest to the cotyledons was discarded to avoid measuring auxin that had diffused from the application site. The amount of radioactivity within each segment was determined using a Beckman LS6500 scintillation counter (Beckman Instruments). For each experiment, 16 to 20 seedlings were used for each treatment, and every experiment was repeated at least three times in total on different days. Because total counts for experiments done on different days varied depending on the batch of radiolabeled auxin, we did not combine the experiments for analysis but present a single representative experiment. Statistical analysis of the repeat experiments showed similar results.

Local Auxin Transport Measurements

Roots were spot inoculated with rhizobia in the emerging root hair zone 4 or 24 h before the start of auxin transport measurements, and the inoculation site marked on the plate. 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. Alternatively, an agar block (2 × 2 × 4 mm^3) containing 1 μM ACC or water (control) agar blocks were placed at the zone of emerging root hairs (Fig. 3A). Just before the start of the experiment, 30 μL of 1 mCi mL^{-1} [^{3}H]IAA (Amersham Biosciences) was diluted in 60 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 2 × 2 × 2 mm^3 donor blocks. Each block contained approximately 16 pmol of [^{3}H]IAA (equivalent to a concentration of 2 μM, of which typically less than 10% is taken up into the roots). Plant roots were cut 6 mm basipetal from the point of spot inoculation and then laid on a plate containing F medium with the basipetal end in contact with a donor 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, covered with aluminum foil, and incubated at 25°C for 6 h. The roots were then cut into two 4 mm long segments, above and below the point of treatment, and the radioactivity in each segment was analyzed by scintillation counting as described above. For each experiment, 20 seedlings were used for each treatment.

Reverse Transcriptase-PCR

Seedlings were grown as described above. Three-day-old seedlings were flood inoculated with *S. meliloti*, and 24 h later the root region (10 mm above and approximately 3 mm below the root tip mark at the time of inoculation) were harvested without the apical 2 mm of root tips. Plant total RNA was extracted using RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. RNA was treated in column with 10 units of RNase-free DNaseI (Invitrogen) at room temperature for 15 min. Following phenol and chloroform extractions and ethanol precipitation, the RNA was resuspended in 20 μL of water and quantified by measuring the A_{260}. After adjusting samples for RNA content, cDNA was synthesized and amplified using the SuperScript one-step reverse transcriptase (RT)-PCR with Platinum *Taq* kit (Invitrogen) from 0.05% RNA following the manufacturer's instruction. PCRs were performed in a thermal cycler at 94°C for 2 min followed by a total of 30 cycles as follows: denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. Primers used for Mt*PIN1* and Mt*PIN2* were according to Schnabel and Frugoli (2004). Primers used for actin (TC107326) were 5′-CAG CCC ACT GGA TGT CTG TA (forward) and 5′-AGC GCA AAT TGA AGA TAC CG (reverse). RT-PCR products were separated on 2% (w/v) agarose gels in 1× Tris-acetate-EDTA buffer and visualized by staining with ethidium bromide. The intensity of each product (percent volume) was determined using Image Master 2D Platinum version 5.0 software (Amersham Bioscience) after converting them into negative images in TIFF file format. This experiment was repeated three times with different batches of plants.

Hormones and Inhibitor Response

IAA, IBA, 2,4-D, and NAA were purchased from Sigma. NPA was purchased from Casei. For stock solutions, 100 μmol IAA, IBA, 2,4-D, and NAA were dissolved in 1 mL of 50% ethanol. NPA was dissolved in 50% methanol. All hormone treatments, including solvent-only controls, received the final concentration of 0.002% ethanol or methanol in the medium. All stocks were filter sterilized and the appropriate amount of hormone was added to the agar before pouring the medium onto plates.

Statistical Analysis

Statistical analysis was carried out with InStat, version 3.06 (Graphpad Software). All data were tested for normality before analysis. Two-way ANOVA was calculated with Genstat for Windows (version 4.2, fifth edition, Lawes Agricultural Trust).

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