

Root and Nodulation Phenotypes of the Ethylene-Insensitive *Sickle* Mutant of *Medicago truncatula*

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The *sickle* (*skl*) mutant of the model legume *Medicago truncatula* is an ethylene-sensitive mutant that have a ten-fold increase in nodule numbers. The nodulation and root phenotypes of the *skl* mutant were investigated and further characterised. The *skl* mutant had longer roots than the wild type, but when inoculated with *Sinorhizobium*, its root length was reduced to the level of wild type. Furthermore, lateral root numbers in uninoculated *skl* were similar to those in uninoculated wild type. However, when the root tips were decapitated, fewer lateral roots formed in *skl* than in wild type. Nodule numbers of the *skl* mutant were significantly reduced by low nitrate concentration (2.5 mM). These results suggest that *skl* mutant has alterations in both root and nodule development.

Key words: *sickle* mutant, ethylene insensitive, *Medicago truncatula*

INTRODUCTION

Nodule development is tightly controlled by the host plant. Nodules are formed mostly in the root region between the root tip and elongation zone at the time of inoculation, which is known as the susceptible zone of nodulation (Bhuvaneswari *et al.* 1980, 1981).

Autoregulation of nodulation (AON) is one of the mechanisms controlling the formation of nodule numbers in younger root tissues (Kinkema *et al.* 2006). Mutants defective in autoregulation allow many new nodules to grow in newly developing roots, resulting in an enhanced nodulation or so-called supernodulation phenotype (Caroll *et al.* 1985b; Sagan & Duc 1996; Wopereis *et al.* 2000; Penmetsa *et al.* 2003). The gene regulating autoregulation has been identified in several legumes and encodes a leucine-rich repeat receptor-like kinase (Krusell *et al.* 2002; Searle *et al.* 2003; Schnabel *et al.* 2005). The autoregulation mutants are able to nodulate in the presence of high nitrate concentrations (Caroll *et al.* 1985a,b; Wopereis *et al.* 2000; Kawaguchi *et al.* 2002). This mutation also has pleiotropic effects on other aspects of plant development as shown in the root phenotype of the *har1-1* mutant of *Lotus japonicus* and the *sym29* mutant of *Pisum sativum* and the *sunn* (super numerary nodules) mutant of *M. truncatula* (Sagan & Duc 1996; Wopereis *et al.* 2000; van Noorden *et al.* 2006). These mutants have short root systems and altered numbers of lateral roots. Grafting studies on these mutants determined that autoregulation is shoot controlled (Delves *et al.* 1986; Jiang & Gresshoff 2002; Krusell *et al.* 2002; Penmetsa *et al.* 2003).

Other endogenous signals that control nodule development are phytohormones. Ethylene has a negative

role in the nodulation of several legumes and is involved at various stages of nodule development (Guinel & Geil 2002; Ferguson & Mathesius 2003; Mulder *et al.* 2005). Consistent with the negative role of ethylene in nodulation, the ethylene-insensitive mutant of *Medicago truncatula*, *sickle* (*skl*), has a hypernodulation phenotype and an increased number of sustained infection threads (Penmetsa & Cook 1997). In contrast to autoregulation mutants, the hypernodulation phenotype of *skl* is root controlled (Prayitno *et al.* 2006).

It is not known whether the *skl* mutant has pleiotropic effects other than its insensitivity to ethylene, such as found in autoregulation mutants. Because the *skl* mutant is defective in ethylene signalling pathway ortholog of Arabidopsis EIN2 (Penmetsa *et al.* 2008), it is likely that the *skl* mutation has an effect on some aspects of ethylene-mediated plant growth, such as adventitious and lateral roots formation. It has been known that ethylene mediates wound-stress response in plants. Decapitation of the root tip induces ethylene-mediated stress responses from wounding (O'Donell *et al.* 1996) and induces lateral root formation (Aloni & Plotkin 1985). Conversely, the formation of adventitious roots in cuttings of the ethylene-insensitive *Never-ripe* (NR) tomato is significantly reduced compared to that of the wild type (Wilkinson *et al.* 1997).

This paper reports the effects of *skl* mutation on the aspects of root growth and nodulation other than those already described by Penmetsa and Cook (1997). These aspects include primary root growth and lateral root formation, nodule numbers and nodule distribution along the root, and the nodulation response to nitrate. Results presented here showed that the *skl* mutant displays pleiotropic effects on root growth and nodulation.

MATERIALS AND METHODS

Plant and Bacterial Growth Conditions. Seeds of *skl* mutant were obtained from Prof. Douglas R. Cook (Penmetsa & 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 several washes, seeds were incubated on nitrogen-free Fåhræus agar medium (Fåhræus 1957) in the dark at 4 °C for 2 days to break their dormancy. A drop of sterile water was applied to each seed to prevent the seeds from drying. 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 Fåhræus agar medium. The seedlings were incubated vertically in the growth chamber with photon flux density of 90 $\mu\text{mol m}^{-2}\text{s}^{-1}$, and 16 h of light per day at 20 °C for 2 days. After 2 days incubation, the seedlings were transferred to fresh Fåhræus plates, and incubated in the same growth chamber. The seedlings were flood-inoculated at the root tips with 5 μl of diluted *Sinorhizobium* suspension 24 h later. The positions of the root tips at the time of inoculation (RT_0) were marked on the plates to provide an initial point of measurement for root growth and nodule position. Root growth was measured from RT_0 to the root tip using a ruler at certain time points.

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 (OD600) of 0.1 or approximately 10^7 cells/ml. As controls, roots were inoculated with an equivalent amount of diluted Bergensen's modified medium.

Decapitated Root Experiments. Roots of *skl* and wild-type plants were decapitated 5 mm from the root tip, and grown on Fåhræus agar medium. Lateral roots (LRs) emerging from the decapitated primary root were recorded at 14 days post decapitation. To examine the effect of IAA on lateral root formation in *skl*, an agar block (5 x 10 x 5 mm) containing 10 μM IAA was placed on the hypocotyls immediately after root tip decapitation. For control treatments, an agar block without IAA was placed on another set of hypocotyls.

RESULTS

The Inoculated *skl* Mutant has a Reduced Root Growth. Uninoculated *skl* plants had a longer primary root growth (PRG) than uninoculated wild-type plants (Figure 1). This difference was observed as early as 3 days after initial measurement (Student's *t*-test, $P < 0.01$). At 9 days after initial measurement, the PRG of uninoculated *skl* was double that of uninoculated wild type. Inoculated *skl* roots were then analysed to see whether their growth was perturbed as a result of the hypernodulation phenotype. Inoculation had no effect on the PRG of wild type (Figure 1). In contrast, inoculation reduced the PRG of *skl* to a length comparable to wild

type. This inoculation-reduced PRG was observed until the experiment ended at 21 days post inoculation (DPI).

Altered Lateral Root Formation in Decapitated Roots of the *skl* Mutant. Alteration of lateral root (LR) phenotypes is commonly found in hypernodulation mutants. Therefore, the effect of *skl* mutation on LR formation was examined. Under non-symbiotic conditions, LR formation in *skl* was not significantly different to that in wild-type roots at 21 day of growing, with the average number of 4.9 and 3.2 lateral roots per plant, respectively (Student's *t*-test, $P = 0.074$, $n = 20$).

It has been established that ethylene mediates wound-stress response in plants. Since intact seedlings of the *skl* mutant showed no alteration in LR formation, the effect of *skl* mutation on the number of LR in decapitated seedlings (wound stress) of *M. truncatula* was investigated. Decapitation of the root tip induced the formation of LRs in wild-type and *skl* plants mostly at the cutting site. The number of LR formed in *skl* at 7 days after decapitation (DAD) was only 37% of the wild-type plants, and this proportion was slightly reduced at 14 days (35%). LR numbers of the wild-type and *skl* plants at 14 DAD were 3.7 and 1.3, respectively (Figure 2a). Initially, *skl* plants had a significantly shorter LR length than wild-type plants (35% of the wild-type) at 7 DAD (Figure 2b). Later at 14 DAD, its LR length was not significantly different from that found in the wild-type plants. These results suggest that a defect in ethylene signalling such as found in the *skl* mutant, causes a reduction in LR formation after wounding and a delay in LR elongation during early stage of LR development (7 DAD).

Auxin is known to stimulate lateral root formation in intact plants and cuttings. Since the number of LR was reduced in the decapitated roots of *skl*, the *skl* mutant may have an altered auxin response for the induction of LR formation after wounding. To test this, roots of *skl* and wild-type plants were decapitated 5 mm from the root tip, and grown on Fåhræus medium in the absence or

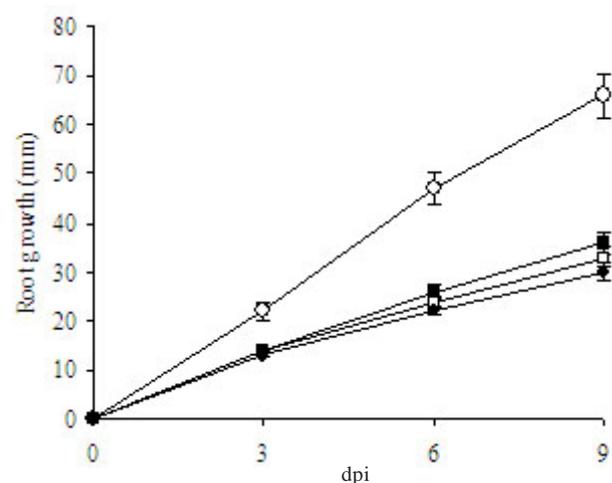


Figure 1. Root growth of A17 (wild type) and *skl* mutant. dpi = days post inoculation. Values are the mean \pm SE of 15 plants. \square A17, \blacksquare A17 + Sm1021, \circ *skl*, \bullet *skl* + Sm1021.

presence of IAA (Figure 3a). LRs emerging from the decapitated primary root were recorded at 14 DAD. IAA induced LR formation in the decapitated roots of *skl* and wild type to a similar extent (1.7 and 1.6 fold increase, respectively; Figure 3b). To determine LR distribution in the decapitated primary roots, the distance of LRs from the cutting site was measured and compared between wild type and *skl*. As shown in Figure 3c, in the absence of

IAA, the decapitation of root tips induced LR formation in wild type and *skl* close to the cutting site. The enhancement of LR number following IAA treatments in both genotypes was mainly due to the increase of LR number toward the hypocotyl (basipetal). This was more pronounced in wild type, where the marked increase of LRs was found in the zone of 1-15 mm from the cutting site (Figure 3c). Although there was an increase in basipetal LR number in *skl*, the distribution pattern of LR in the decapitated primary roots was similar regardless of IAA treatment, with the highest number of LR at the cutting site (Figure 3c). These results demonstrate that when the root tip is decapitated, the *skl* mutant has an altered response to IAA-stimulated LR formation in the primary root.

Nodule Numbers and Nodule Distribution in the *skl* Mutant. Nodule numbers and nodule distribution in *skl* were examined at 21 DPI. In wild type, the average number of nodules per plant at 21 DPI was 3.2. These nodules were formed in the susceptible zone of nodulation, which was approximately 10 mm above RT_0 and 20 mm below RT_0 (Figure 4a,b). In *skl*, a higher number of nodules was also observed in the susceptible zone with the average numbers per plant being 24.1. In this region, nodules formed a cluster with reduced size of each nodule (Figure 4c,d). In a small percentage of plants (two in fifteen plants), two clusters were present along the primary root of *skl*, one of which was found beyond the susceptible zone, or approximately in the root region between 25 to 35 mm below RT_0 (Figure 4e).

The Nodulation of the *skl* Mutant in the Presence of High Nitrate Concentration. Nitrate tolerance is an important characteristic of supernodulation mutants defective in autoregulation of nodulation. To investigate the nodulation response of *skl* to nitrate, various levels of nitrate were tested. Nitrate concentration as low as 2.5 mM sharply reduced nodule numbers in wild type to 12% of the control treatment (Figure 5). The same nitrate concentration also decreased nodule numbers in *skl*, but not as severely as in the wild type, i.e. to 56% of the

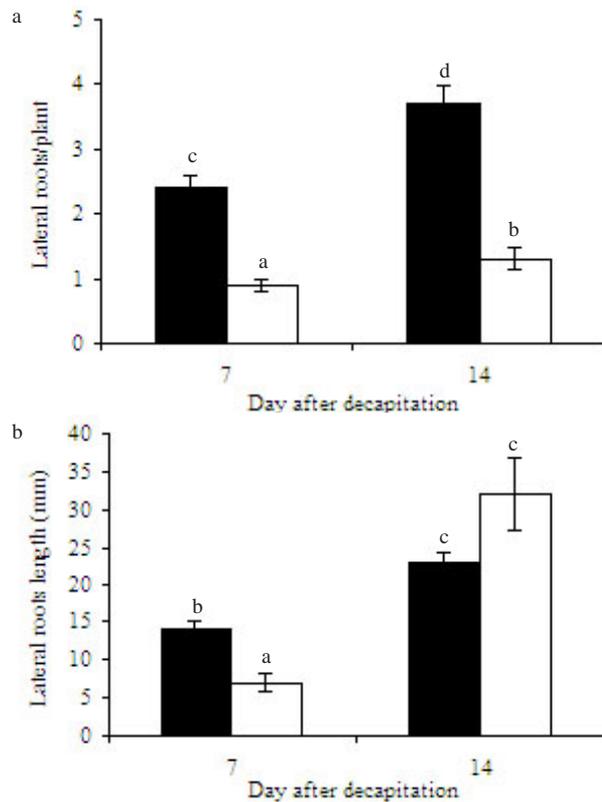


Figure 2. Lateral root formation after root tip decapitation. (a) lateral root numbers per plant. (b) lateral root length. Values are mean \pm SE of the average lateral root length from 27-32 plants. Different lower case letters indicate significant difference between treatments according to the non-parametric Mann-Whitney test at $P < 0.05$. ■ WT, □ *skl*.

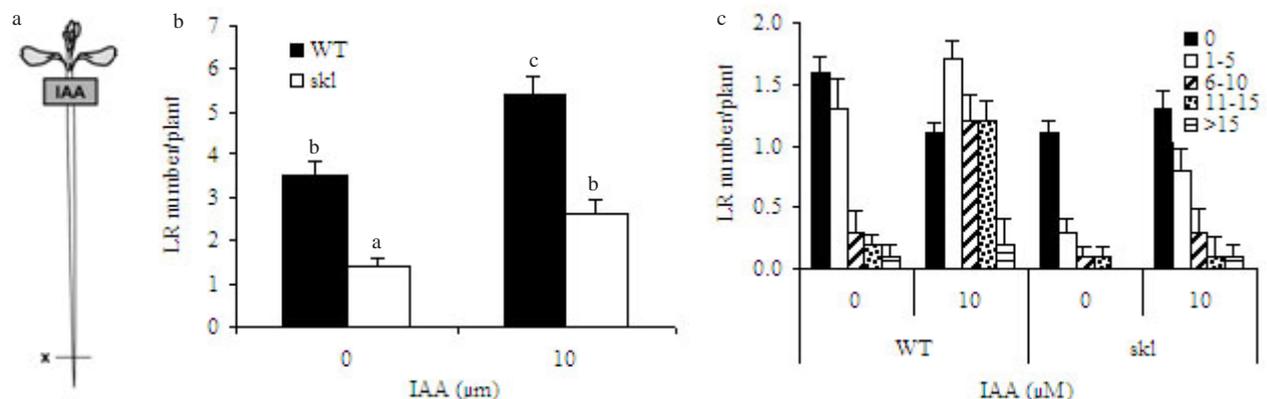


Figure 3. Lateral root formation at 14 days after root-tip decapitation. (a) a schematic diagram of the experiment. The root was decapitated 5 mm from the root tip (X), and an agar block (5 x 10 x 5 mm) containing 10 μ M IAA was placed on the hypocotyl. The plants were incubated on agar medium. (b) effect of 10 μ M IAA on lateral root numbers. Bars with different lowercase letters are significantly different according to the non-parametric Mann-Whitney test, $P < 0.05$ ($n = 15-17$, mean \pm SE). (c) distribution of lateral roots on the decapitated primary roots. The decapitated primary roots were divided into five zones, at the cutting site (0 mm), 1-5, 6-10, 11-15 and >15 mm above the cutting site (Mean \pm SE, $n = 15-17$).

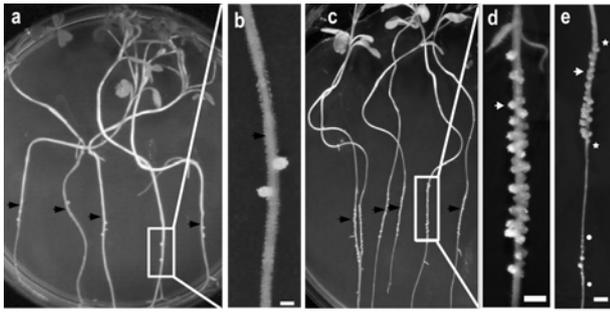


Figure 4. Nodule formation and distribution at 21 DPI. (a) nodule formation in wild type. (b) nodulation region boxed in (a) is highlighted. (c) nodule formation in *skl*. (d) a cluster of nodules boxed in (c) is highlighted. Black or white arrows indicate RT_0 . Bars = 1 mm. (e) two clusters of nodules formed in *skl* in the region between white stars and white dots. White arrow indicates RT_0 . Bar = 2.5 mm.

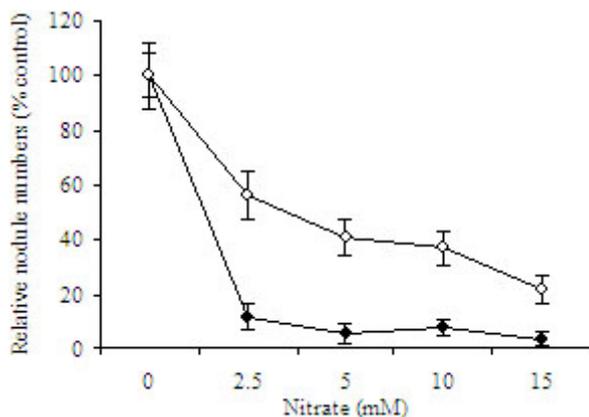


Figure 5. Nodule numbers in response to nitrate at 21 DPI. Values are normalised to untreated control plants (mean \pm SE of 20 plants). The average nodule numbers of wild-type and *skl* plants at 0 mM nitrate are 4.7 and 19.6, respectively. \bullet WT, \circ *skl*.

control plants. Higher nitrate concentrations from 5 to 20 mM further reduced nodule numbers in *skl* to 22%, while nodule numbers in wild type were reduced to 5% of the untreated control (Figure 5). These results indicate that nitrate inhibits nodulation in the *skl* mutant; however the *skl* mutant is more tolerant to nitrate than the wild type.

DISCUSSION

Root Growth Inhibition of *skl* Plants by *Sinorhizobium*.

Results reported here showed that the *skl* mutation had an effect on root growth. In the absence of the symbiotic partner, *skl* roots were longer than wild-type roots (Figure 1). Other features of uninoculated *skl* roots have been described in previous reports including an increase of cell elongation and reduction of root diameter (Prayitno & Mathesius 2010) and root hair elongation (Oldroyd *et al.* 2001). Together, these root phenotypes of the *skl* mutant showed characteristics of ethylene insensitivity. In contrast, the primary root growth in *skl* mutant was reduced in the presence of rhizobia. In wild type, inoculation did not reduce the primary root growth.

Because *Sinorhizobium* inoculation induced the hypernodulation phenotype in *skl*, these results suggest that the root growth inhibition of *skl* upon inoculation is correlated to its hypernodulation response. Ethylene insensitivity is causal to hypernodulation phenotype of *skl*, and the hypernodulation response then reduces the primary root growth.

Reduced plant growth following inoculation has been previously reported in supernodulating mutants *har1-1* of *L. japonicus* and *nts382* of soybean (Carroll *et al.* 1985a; Wopereis *et al.* 2000). However, the root retardation in *har1-1* is more extreme than in *skl*, and is observed both in the absence and presence of rhizobia. In addition, the root growth reduction in *nts382* occurs at a later stage, when the roots have become extensively nodulated. Therefore, it appears that root growth inhibition in supernodulating mutants following inoculation is a common phenomenon in legumes. This inhibition of root growth in supernodulating mutants could be a result of an increase in photosynthate allocation into the nodulation region at the expense of the root growth (Wopereis *et al.* 2000), or changes in hormonal response or transport such as auxin (van Noorden *et al.* 2006).

Lateral Root Formation in the Ethylene-Insensitive Mutants after Root Tip Decapitation. In transgenic plants constitutively expressing the Arabidopsis *etr1-1*, the adventitious root formation of the cuttings was severely inhibited as a result of its reduced sensitivity to ethylene (Wilkinson *et al.* 1997; Shibuya *et al.* 2004), suggesting that the endogenous auxin, which is thought to be the primary inducer of lateral root formation, requires ethylene signalling to stimulate the formation of adventitious roots in cuttings. Results presented here demonstrated that the ethylene-insensitive *skl* mutant has also an altered lateral root formation after root-tip decapitation. The reduced capacity of the *skl* mutant to form lateral roots after root-tip decapitation is similar to that found in the tomato mutant *Never-ripe*, an ethylene insensitive mutant with a mutation in the ETR1 receptor, and to that found in the transgenic petunia line 44,568 plants constitutively expressing the *etr1-1* gene (Clark *et al.* 1997; Wilkinson *et al.* 1997; Shibuya *et al.* 2004). However, lateral root formation in *skl* was more like in the *Never-ripe* tomato plants. In *skl* and *Never-ripe* plants, rooting was not totally inhibited (approximately 50% of the wild-type plants), while in the transgenic petunia line 44,568 rooting was almost totally inhibited (less than 1% of the wild type). The adventitious roots of the *Never-ripe* mutant and transgenic line 44,568 were shorter than the wild-type roots at three weeks after cutting. During early growth of lateral roots, *skl* plants also had a shorter lateral roots than wild-type plants, although later, their lateral root elongation was similar to that of wild type. These differences could be due to the different mutation conferring ethylene sensitivity in different species.

Following root-tip decapitation, LR numbers formed on the *skl* primary roots were significantly lower than those on wild-type roots (Figure 2a). In contrast, intact *skl* plants had comparable numbers of lateral roots to wild-type

plants. These results suggest that the reduction of LR formation in *skl* after root-tip decapitation was correlated with its insensitivity to ethylene. Because auxin transport from the shoot to the root is required for the formation of lateral roots (Casimiro *et al.* 2001), and auxin treatment at the hypocotyl induced basipetal LR formation in wild type (Figure 3b), it is likely that the *skl* mutant has an altered auxin transport or response following root-tip decapitation. A model for the role of ethylene and auxin transport in lateral root formation has been proposed (Aloni *et al.* 2006). In this model, ethylene is produced locally following wounding, flooding or external ethylene application. Local ethylene accumulation locally inhibits auxin transport in the pericycle. Immediately above this inhibition site, newly arriving IAA is accumulated and then stimulates cell division in the pericycle (Aloni *et al.* 2006). Similarly, the effect of the *skl* mutation on auxin transport and auxin response during nodulation has been reported (Prayitno *et al.* 2006). In their report, ethylene signaling negatively affects auxin uptake into the nodulation zone, partially by affecting *PIN1* and *PIN2* gene expression (Prayitno *et al.* 2006).

Nodulation and Nitrate Responses of the *skl* Mutant.

Nodule development is restricted to the susceptible zone of nodulation in roots, which is controlled by the host plant through long distance signalling or known as AON (Kinkema *et al.* 2006). Alteration of this regulation such as found in the AON mutant *nts 382* of soybean resulted in an increase of nodule numbers within the wider zone of nodulation (Carroll *et al.* 1985b). In the ethylene-insensitive *skl* mutant, the increased nodulation occurs within the susceptible zone of nodulation (Figure 4c,d), without an increase in nodulation zone. These results suggest that the hypernodulation phenotype in *skl* is regulated by ethylene that is different from AON.

Nitrate inhibition of nodulation is a common phenomenon among legume species. A supernodulation of soybean, *nts382*, which has a defect in AON, was obtained from screens of plants for nitrate tolerance (Carroll *et al.* 1985a,b). Nodule numbers in this mutant were increased in the presence of 5 mM nitrate compared to wild type (Carroll *et al.* 1985b). Another AON mutant, the *har1-1* mutant of *L. japonicus*, is also insensitive to high concentrations of nitrate (5-15 mM nitrate), with 40-60 nodules developed at 6 weeks after inoculation (Wopereis *et al.* 2000). The wild type of *L. japonicus* only forms a few bumps in the presence of 15 mM KNO₃. Unlike AON mutants, nodule numbers in *skl* were reduced in the presence of low nitrate concentration (2.5 mM), although the nodulation in *skl* was less sensitive to the inhibitory effect of nitrate than that in wild type (Figure 5). Previous reports have suggested that ethylene might mediate the suppression of nodulation by nitrate, since nitrate increased ethylene production in alfalfa roots, and L- α -(2-aminoethoxyvinyl)-glycine (AVG) could overcome the inhibitory effect of nitrate by the inhibition of ethylene biosynthesis (Ligero *et al.* 1986; Ligero *et al.* 1991). In contrast, the nodulation in *skl*, a mutant having a defect in ethylene perception, was suppressed by low nitrate

concentration (Figure 5). In addition, two lines of evidence in pea have suggested that ethylene might not be involved in nitrate inhibition of nodulation (Lee & LaRue 1992). Lee and LaRue (1992) showed that addition of Ag⁺, an ethylene perception inhibitor, were not able to reverse the inhibitory effect of nitrate on nodulation, and that the block of nodulation by nitrate occurred at an earlier stage than that by ethylene.

Overall, this study has shown that the hypernodulating *skl* mutant shows alterations in both root and nodule development. Since the *skl* mutant is defective in ethylene signaling, this mutant can be used to further analysis the role of ethylene in stages of nodule development at the tissue, cellular and molecular level to gain better understanding of plant development.

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