

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 *sun* (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 Fåhreaus agar medium (Fåhreaus 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 Fåhreaus 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 Fåhreaus 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 Fåhreaus 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 × 10 × 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 Fåhreaus medium in the absence or

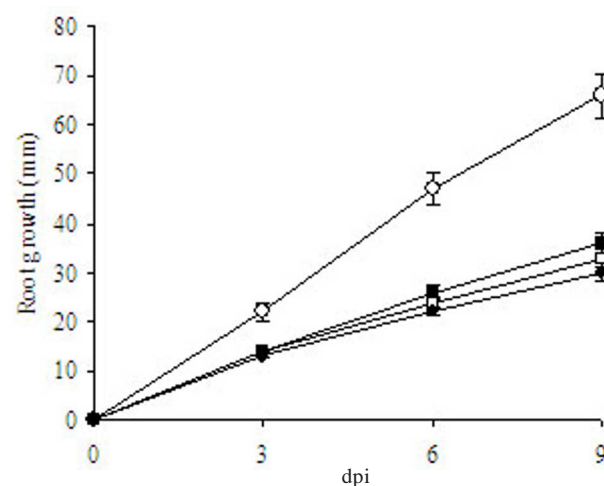


Figure 1. Root growth of A17 (wild type) and *skl* mutant. dpi = days post inoculation. Values are the mean \pm SE of 15 plants. □— A17, ■— A17 + Sm1021, ○— *skl*, ●— *skl* + Sm1021.

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