Review article

Involvement of polyamines in the root nodule regulation of soybeans (*Glycine max*)

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Abstract: In leguminous plants, infection with rhizobia leads to the formation of root nodules that fix atmospheric nitrogen and supply it as ammonium to the host plant cells. The formation of nitrogen-fixing nodules is nutritionally beneficial, but excessive nodule production appears to be detrimental to the host legumes because of the resultant over-consumption of photosynthetic products. Therefore, the number of root nodules in leguminous plants is tightly regulated by shoot-root signaling known as a feedback or autoregulation of nodulation. Recently, we found that foliar application of polyamines and an effective inhibitor of polyamine biosynthesis regulates the number of nodules in soybean plants. In this review, we discuss the possible function of polyamines as a systemic regulator of nodule formation.

Keywords: autoregulation, *Glycine max* L. (Merr.), nodule, polyamine, supernodulating mutant

Nodule formation in leguminous plants

Root development in plants is a complex process involving a high degree of morphological plasticity, which reflects inherent mechanisms of adaptation to highly variable environmental conditions. Physiological experiments suggest that both local and systemic regulation are involved in the determination of root plasticity. In addition to its role in obtaining sources of nutrients and water, an important role of root developmental plasticity is to provide plants with the ability to recognize and respond to diverse biotic signals from soil microorganisms.

Leguminous plants form root nodules as part of a symbiotic association with rhizobia. Nodule organogenesis is activated in response to lipochito-oligosaccharides, specific signal molecules called Nod factors which are synthesized by compatible strains of rhizobia (Lerouge et al. 1990, Truchet et al. 1991). Nodule structures allow the fixation of atmospheric nitrogen, but are sometimes energetically expensive to develop while still maintaining the normal growth of the host leguminous plant since the process of nitrogen fixation requires a large quantity of photosynthetic products (Fujikake et al. 2003, Ito et al. 2006). Therefore, the number of nodules is controlled by a self-regulating mechanism known as autoregulation, in which previous nodulation events inhibit the over-production of nodules on young root tissues (Pierce and Bauer 1983, Caetano-Anolles et al. 1991). Grafting experiments between wild-type lines and supernodulating mutants with a mutation in the supernodulation genes and lacking the self-control systems have revealed that autoregulation consists of two long-distance signals: a root-derived signal and an autoregulation signal (Carroll et al. 1985, Gremaud and Harper 1989, Akao and Kouchi 1992). The former is generated in the roots in response to rhizobia, while the latter is produced in shoots upon receiving the root-derived signal (Caetano-Anolles et al. 1991, Sheng and Harper 1997, Oka-Kira and Kawaguchi 2006). Recently, it was determined by positional cloning that the gene involved in autoregulation signaling in *Lotus japonicus* and soybeans encodes a putative receptor kinase (Krusell et al. 2002, Nishimura et al. 2002, Searle et al. 2003), however, the principal signal molecule(s) involved in the communication between the shoot and root remains to be determined.

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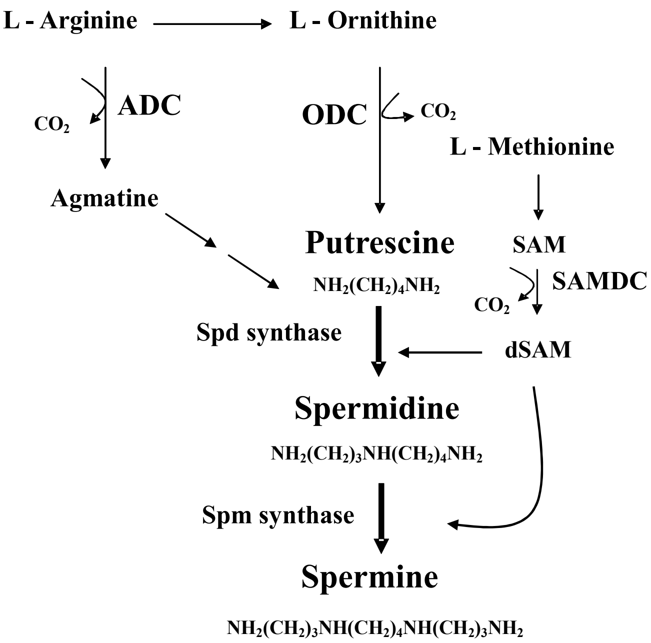


Fig. 1. Polyamine biosynthetic pathways in higher plants. ADC: arginine decarboxylase, ODC: ornithine decarboxylase, SAMDC: *S*-adenosylmethionine decarboxylase, SAM; *S*-adenosylmethionine, dSAM: decarboxylated *S*-adenosylmethionine.

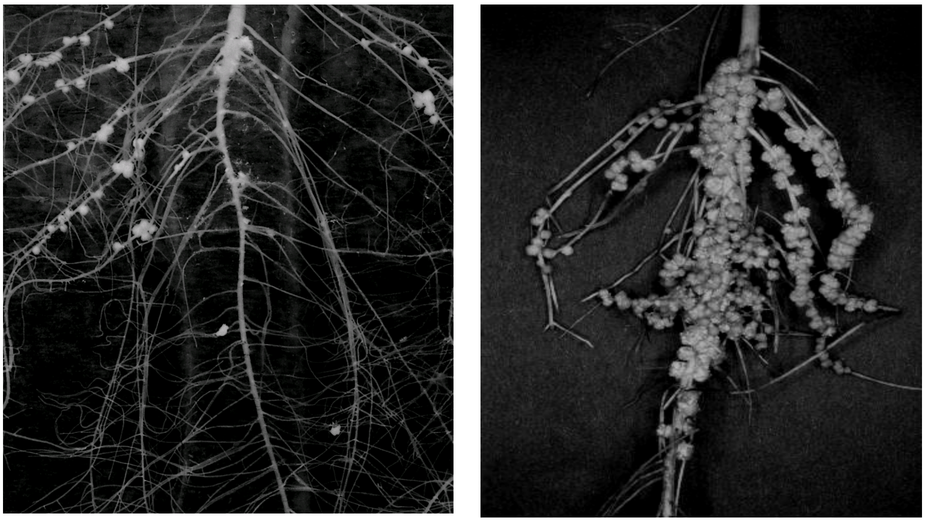
Biosynthesis of polyamines and their role in plants

Polyamines, that is, spermidine (Spd), spermine (Spm) and their obligate precursor putrescine (Put), are ubiquitous in a wide range of organisms from bacteria to plants and animals. They are small, positively charged aliphatic amines and therefore bind to negatively charged molecules including nucleic acids, acidic phospholipids and proteins (Cohen 1998).

The biosynthetic pathways for polyamines have been well established in both eukaryotic and prokaryotic organisms (Fig. 1). Eukaryotic cells synthesize Put directly from ornithine through the activity of the enzyme, ornithine decarboxylase (ODC; EC 4.1.1.17) (Tabor and Tabor 1984, Pegg 1986, Heby and Persson 1990). In plants, Put is also synthesized indirectly from arginine via agmatine and N-carbamoylputrescine (Smith 1985, Tiburcio et al. 1990). The biosynthesis of Spd and Spm is catalyzed by Spd synthase (EC. 2.5.1.16.) and Spm synthase (EC. 2.5.1.22.) by the addition of an aminopropyl moiety to the diamine, Put and the triamine, Spd, respectively (Kumar et al. 1997, Malmberg et al. 1998). *S*-adenosylmethionine (SAM) decarboxylase (SAMDC; EC 4.1.1.50) is a key enzyme in the biosynthesis of both Spd and Spm from Put and its activity has been shown to be rate-limiting in this pathway (Slocum et al. 1984, Tabor and Tabor 1984). SAMDC is also known to influence the rate of biosynthesis of ethylene (Apelbaum et al. 1985) since SAM is a common precursor in both biosynthetic pathways (Galston and Sawhney 1990).

Polyamines have been implicated in a variety of plant growth and developmental processes involving cell proliferation and differentiation, morphogenesis, development and stress tolerance (Evans and Malmberg 1989, Galston and Sawhney 1990). Additionally, there is accumulating evidence that infections of various pathogenic fungi and viruses bring about dynamic changes in polyamine metabolism not only in the infected cells but also in other regions of the host plants (Walters 2000, Pérez-Amador et al. 2002).

Previously, we reported that the root nodules of leguminous plants accumulate polyamines to levels that are five to ten times higher than in other organs (Fujihara et al. 1994). During the course of the polyamine analysis of a variety of leguminous plants, profiles of nodule polyamine composition were found to be basically similar within the same species as far as root nodules were collected from the plants at physiologically similar growth stage (Fujihara et al. 1994). Interestingly, however, root nodules of supernodulating mutants of soybean plants showed apparently different polyamine composition from those of their parent lines and other soybean cultivars, suggesting an abnormality of polyamine metabolism in supernodulating mutants. In view of the above finding, we decided to investigate the effect of polyamines on root nodule formation using the supernodulating mutant En6500 (Akao and Kouch 1992) and its parent line cv.



Enrei **En6500**

Fig. 2. Nodulation of the soybean (*Glycine max*) wild-type cv. Enrei and the supernodulating mutant En6500.

Enrei (Fig. 2).

Regulation of nodule formation by polyamines

In order to determine the polyamine content of soybean plants, we compared the polyamine composition in different tissues of field-grown cv. Enrei and supernodulating mutant En6500 in the vegetative stage. The Put levels of the leaves, roots and nodules in En6500 were higher than those in Enrei, but the Spd and/or Spm levels in En6500 were consistently lower than those in Enrei (Fig. 3). These data imply that Spd biosynthesis from Put is suppressed in En6500. This difference in tissue polyamine composition, that is, the low Spd/Put ratio in En6500, was also found in the early reproductive stages (data not shown).

Sato et al. (1997) showed that the number of nodules on adventitious roots formed from excised leaflets of the hypernodulating soybean mutants was greater than that of wild type. Foliar treatment with biological materials seems to be an effective method to test their role in autoregulation. Thus, to determine the possible involvement of shoot polyamines in nodule regulation, we next examined the influence of the foliar application of polyamines on root nodule formation (Terakado et al. 2006). In this experiment, cv. Enrei and its supernodulating mutant En6500 were grown in plastic growth pouches, set in growth chambers and supplied with N-free nutrient solutions. The foliar application of Put at one day after rhizobia inoculation did not significantly influence nodule production in either cv. Enrei or En6500 (Fig. 4). However, the foliar application of Spd or Spm led to a significant reduction of nodule formation in En6500

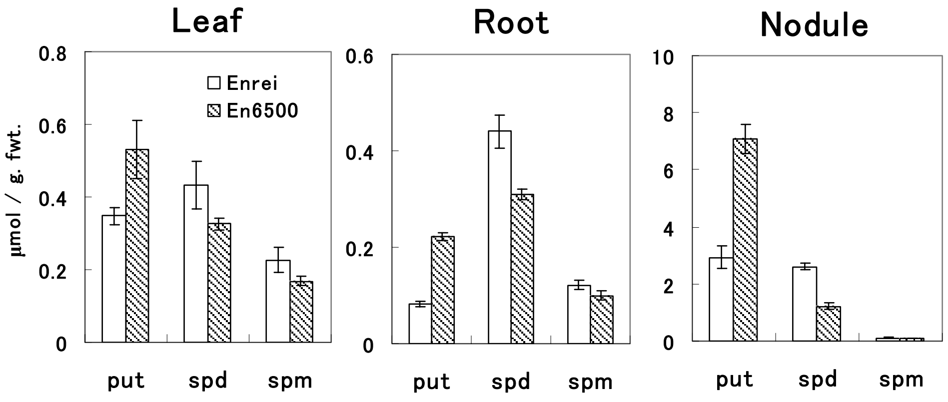


Fig. 3. Polyamine contents of wild-type cv. Enrei and supernodulating mutant En6500. Each value represents the mean (±SD) of at least three different samples.

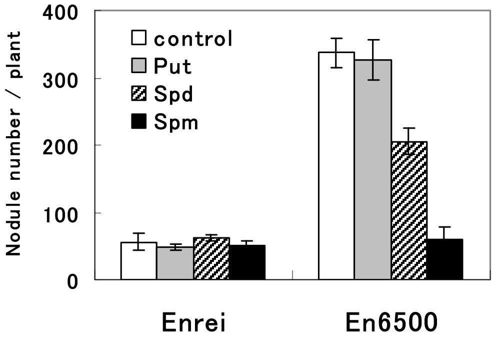


Fig. 4. Nodulation of wild-type cv. Enrei and supernodulating mutant En6500 subjected to the foliar application of Put, Spd or Spm. Two μmol of polyamines (Put, Spd or Spm) and 0.5 mg triton per mL deionized water were applied at a dose of 0.1 mL per plant on the surface of all leaves of cv. Enrei and En6500 at 1 day after rhizobia inoculation. The control solution contained triton.

(Fig. 4). Specifically, Spm suppressed the number of nodules in En6500 to the same level as that of cv. Enrei.

The size of root nodules closely relates to the number of nodules formed on the root, and excessive production of root nodules generally results in the reduction of nodule size under the limited supply of photosynthetic products to the roots. Foliar treatment with Spd and/or Spm restored the nodule size of En6500 to almost the same size as the wild type, possibly due to the suppression of over production of root nodules in En6500. We also observed that the foliar application of Spd or Spm promoted shoot growth of En6500 while resulted in a 30% reduction of the total length of the roots (data not shown).

MDL74038 is a synthetic compound developed as a specific inhibitor of SAMDC, a key enzyme of polyamine biosynthesis (Wright et al. 1991). This chemical blocks Spd biosynthesis by preventing the supply of the aminopropyl group from SAM to Put. To examine whether the inhibition of Spd synthesis in the shoot affects root nodulation, the leaves of Enrei were treated with MDL74038. After 72 hrs, the Spd level in the leaves of Enrei was found to have decreased by 35% and nodulation on the roots was greatly accelerated (Fig. 5) although the number of nodules did not reach to the level of En6500, possibly due to the short duration of the inhibitory effect of MDL74038 in the leaves.

As has already been described (Carroll et al. 1985, Gremaud and Harper 1989, Akao and Kouchi 1992), the growth rate of supernodulating mutants is generally far below that of their parent cultivars (Fig. 6). Interestingly, we observed that the stem elongation of Enrei was apparently inhibited, as in En6500, by the treatment with MDL74038 (Fig. 7). It is postulated that signals related to the autoregulation might not be specific only to the nodulation but also be a part of general plant development (Beveridge et al. 2007, Buzas and Gresshoff 2007). Ito et al. (2007, 2008) recently suggested that the root-derived autoregulation signal induces the leaf expansion, especially the cell proliferation of leaf blade. Inhibition of the root growth and stimulation of the shoot growth, both of which are induced by the foliarly applied polyamines as described above, might closely link to the suppression of nodule formation.

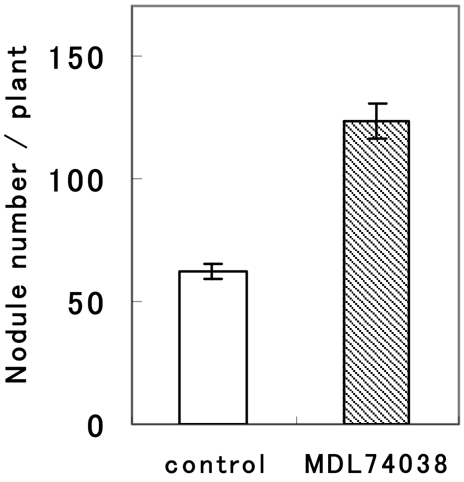


Fig. 5. Nodulation of wild-type cv. Enrei subjected to the foliar application of the Spd-biosynthesis inhibitor MDL74038. One μmol MDL74038 and 0.5 mg triton per mL deionized water were applied at a dose of 0.1 mL per plant on the surface of all leaves of cv. Enrei every second day for 14 days. The control solution contained triton.

Does foliar polyamine translocate to the root in nodule regulation?

Based on the above results, we speculated that foliar polyamines might play a role in nodule regulation. However, it remains to be determined whether the regulatory compound(s) transported from the shoot to the root consist(s) of a specific polyamine or of other compounds induced by the polyamine.

Long-distance transport is one of the factors to take into consideration in order to determine if polyamines are a plant growth regulator or a secondary messenger in root nodule regulation. There are several investigations on the transport of exogenously added polyamines in higher plants. For example, it has been demonstrated that Put absorbed from the leaves and fruitlets of apple tree is partly metabolized to Spd and Spm, and that these are then translocated to other parts (Bagni et al. 1984). Caffaro et al. (1993) also

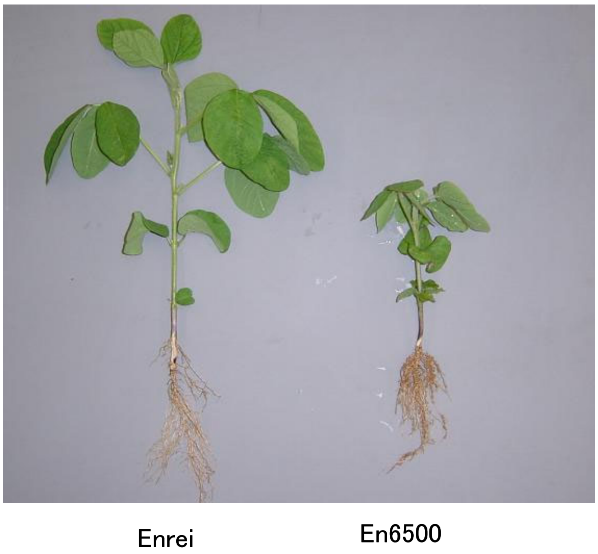


Fig. 6. Vegetative growth of soybean plants. Wild-type cv. Enrei and supernodulating mutant En6500 were grown in the experimental field and harvested at 29 days after sowing.

report that a continuous supply of ^{14}C-labeled Spd to the soybean leaf was translocated throughout the plant body. On the other hand, Ohe et al. (2005) show that exogenous Put was easily absorbed from the root, converted to Spd and Spm, and translocated to the upper parts in soybean seedling. In order to determine whether foliarly administered polyamine translocates in soybean plants in our experimental systems, we applied ^{15}N-labeled Put to the leaves (281.4 μg ^{15}N /g DW) and investigated the time course of ^{15}N incorporation into polyamine molecules in different organs using gas chromatography/combustion/mass spectrometry (J. Terakado unpublished data). After 24 hrs of the treatment with ^{15}N-Put, ^{15}N contents of Put, Spd, and Spm molecules in the leaf were 21.5, 5.6, and 2.1 μg/g DW, respectively. It is expected that the ^{15}N detected in Spd and Spm could be derived from ^{15}N-Put absorbed by the treated leaf. In the root, however, the amount of ^{15}N in each polyamine was very low even after 72hrs (^{15}N contents of Put, Spd, and Spm molecules in the root were 0.04, 0.02, and 0.001 μg /g DW, respectively). Thus, our study strongly indicated that foliarly applied polyamine and its metabolites are only slightly transported to the underground parts.

The functional role of leaf Spd and/or Spm in root nodule regulation remains unclear at present. However, it is possible that remarkable changes in polyamine composition or the pool size of polyamines in the shoot induce significant biochemical, physiological or morphological changes in the roots. Infections of various pathogenic fungi and viruses are known to induce dynamic change in the polyamine metabolism, not only in the infected cells but also in other regions of the host plants (Walters et al. 2002, Pérez-Amador et al. 2002). Such metabolic changes in polyamines may be associated with a particular defense mechanism of the plants, generally known as hypersensitive response or systemic acquired resistance (Takahashi et al. 2003). In TMV-infected tobacco plants, Spm is known to accumulate in intercellular spaces and induce pathogenesis related proteins during hypersensitive response (Yamakawa et al. 1998, Takahashi et al. 2003). As regards the function of accumulated polyamine in tobacco leaves, Yoda et al. (2003) currently hypothesized that the generation of H_2O_2 from Spd and /or Spm by the action of polyamine oxidase might trigger a defense signal. Considering the postulated role in a series of signal transductions, polyamines, especially Spd and/or Spm, in the leaves might play a systemic role, not only in disease resistance but also in nodule regulation without translocation to the roots.

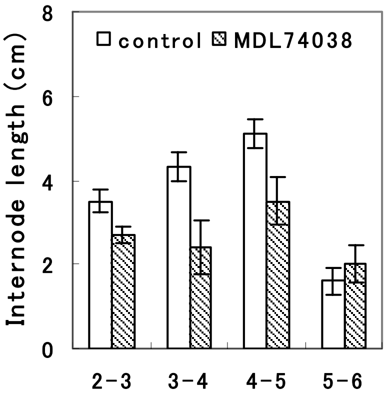


Fig. 7. Effect of foliar treatment with the Spd-biosynthesis inhibitor MDL74038 on internode growth in wild-type cv. Enrei.

Cross-talk between polyamines and plant hormones

Polyamines are known to mediate hormone-induced plant responses in many physiological processes. It has been demonstrated that exogenous treatment with plant hormones changes tissue polyamine contents and/or expresses genes that are involved in the biosynthetic pathways of polyamines (Ghachtouli et al. 1996, Biondi et al. 2001, Hanzawa et al. 2002, Pérez-Amador et al. 2002, Walters et al. 2002). Brassinosteroids (BRs), a group of recently identified plant hormones, are able to control vascular differentiation, photomorphogenesis, fertility, root growth and seed germination (Bishop and Yokota 2001). Mutants lacking BR biosynthesis display dwarf phenotypes and can be restored to a wild-type phenotype by the external application of brassinolide (BL), a BR (Li et al. 1996, Nomura et al. 1997, Bishop et al. 1999). It has also been suggested that BL systemically induces resistance to fungal pathogens and bacterial diseases in higher plants (Nakashita et al. 2003).

Previously, we found that root nodule formation in the supernodulating soybean En6500 was significantly inhibited by the foliar application of BL and, in contrast, the nodule number of the wild-type soybean cv. Enrei was greatly promoted by treatment with brassinazole, an inhibitor of BR synthesis (Terakado et al. 2005). We also observed that foliar treatment with BL not only reduces root nodulation but also changes the endogenous levels of polyamines in En6500, elevating leaf Spd after a transient increase in Put (Terakado et al. 2006). Considering that foliar treatment with polyamines and/or BL reduced root nodulation and induced the shoot growth of En6500 while inhibitors of their synthesis increased root nodulation and suppressed the shoot growth of Enrei, we speculate that BL-induced polyamine synthesis may regulate root nodule formation concomitantly with shoot growth in soybean plants.

Concluding remarks

We found that shoot-applied polyamines, especially Spd and/or Spm, suppress root nodule formation in soybean plants. Likewise, the foliar application of the plant hormone BL not only reduced the nodule number but also changed the polyamine levels in the supernodulating mutant En6500. There have been many other reports that treatments with plant hormones such as methyl jasmonic acid, abscisic acid, salycilic acid or ethylene suppress nodule formation in leguminous plants (Lee and LaRue 1992, Sato et al. 2002, Suzuki et al. 2004, Nakagawa and Kawaguchi 2006). Based on the results of these previous studies, it is probable that cross-talk between polyamines and plant hormones leads to the effective regulation of nodule formation. However, it must also be taken into consideration that exogenous treatments with these plant hormones sometimes induce feedback regulation and/or significant metabolic changes in plants (Fujioka and Yokota 2003). Measurement of the endogenous level of these plant hormones and investigation of the candidate genes which control the number of nodules are necessary to clarify the systemic mechanism of polyamines on nodule formation.

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