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ORIGINAL ARTICLE

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Lack of mycorrhizal autoregulation and phytohormonal changes in the supernodulating soybean mutant *nts1007*

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Abstract Autoregulatory mechanisms have been reported in the rhizobial and the mycorrhizal symbiosis. Autoregulation means that already existing nodules or an existing root colonization by an arbuscular mycorrhizal fungus systemically suppress subsequent nodule formation/root colonization in other parts of the root system. Mutants of some legumes lost their ability to autoregulate the nodule number and thus display a supernodulating phenotype. On studying the effect of pre-inoculation of one side of a split-root system with an arbuscular mycorrhizal fungus on subsequent mycorrhization in the second side of the split-root system of a wild-type soybean (*Glycine max* L.) cv. Bragg and its supernodulating mutant *nts1007*, we observed a clear suppressional effect in the wild-type, whereas further root colonization in the split-root system of the mutant *nts1007* was not suppressed. These data strongly indicate that the mechanisms involved in supernodulation also affect mycorrhization and support the hypothesis that the autoregulation in the rhizobial and the mycorrhizal symbiosis is controlled in a similar manner. The accumulation patterns of the plant hormones IAA, ABA and Jasmonic acid (JA) in non-inoculated control plants and split-root systems of inoculated plants with one mycorrhizal side of the split-root system and one non-mycorrhizal side, indicate an involvement of IAA in the autoregulation of mycorrhization. Mycorrhizal colonization of soybeans also resulted in a strong induction of ABA and JA levels, but on the basis of our data the role of these two phytohormones in mycorrhizal autoregulation is questionable.

Keywords Abscisic acid · Arbuscular mycorrhiza · Autoregulation · Auxin · Glomales · Jasmonic acid · Supernodulation

Abbreviations ABA: Abscisic acid · AMF: Arbuscular mycorrhizal fungus · IAA: Indole-3-acetic · JA: Jasmonic acid · min: minute

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Introduction

Arbuscular mycorrhiza (AM) is a symbiosis between soil-borne fungi and plants. Arbuscular mycorrhizal fungi (AMF) colonize plant roots and improve the P-nutrition of the plant, while the plant is providing the fungus with carbohydrates (Smith and Read 1995). Legume plants can form a symbiosis not only with AMF, but also with nodule forming, N_2-fixing rhizobial bacteria. From the plant's perspective, the development of a symbiotic association such as the rhizobial and the mycorrhizal symbiosis is a beneficial, but also costly process. Thus host plants developed regulatory mechanisms to limit the energy expenses (Caetano-Anollés and Gresshoff 1991; Vierheilig 2004a).

A so-called autoregulatory mechanism is well documented in the *Rhizobium*–legume interaction. Autoregulation of nodulation means that already existing nodules systemically suppress subsequent nodule formation in other parts of the root system (for details see review Caetano-Anollés and Gresshoff 1991). Mutants of some legumes lost their ability to autoregulate the

nodule number and thus display a supernodulating phenotype characterized by the formation of a high number of nodules (e.g. Carroll et al. 1985; Wopereis et al. 2000). Recently, the first genes involved in the autoregulation of nodulation have been identified by map-based cloning in *Lotus japonicus* and soybean. These genes are CLAVATA1-like receptor kinases and seem to be essential parts of the signalling circuit for autoregulation (Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2003). The supernodulating soybean mutant *nts1007* used in this study has a nonsense termination mutation in the kinase domain of GmNARK (*Glycine max* nodule autoregulation receptor kinase; Searle et al. 2003). In *Arabidopsis thaliana*, the *CLAVATA* genes are components of a signalling pathway that is required to restrict the amount of stem cells in the apical shoot and floral meristems. CLAVATA3 (CLV3) has been identified as an extracellular peptide signal that might play a role as short-distance morphogen (Brand et al. 2000; Rojo et al. 2002; Boller 2005).

In the AM symbiosis, a root colonization-regulating mechanism has been recently identified (Vierheilig and Piché 2002; Vierheilig 2004a). Once a critical degree of AM colonization is reached, further root colonization by AMF is suppressed (Vierheilig 2004b). This effect is systemic, i.e. even in parts of the root system of a mycorrhizal plant without root colonization by AMF, further mycorrhization is suppressed (Vierheilig et al. 2000a, b; Vierheilig 2004b). Reminiscent to the autoregulatory mechanism in the rhizobial symbiosis, this mechanism has been termed "autoregulation of mycorrhization" (Vierheilig 2004a).

Recently, it was shown that an established mycorrhizal association systemically suppresses not only further mycorrhization but also nodulation and that nodulation systemically suppresses later mycorrhization (Catford et al. 2003). An inhibition effect was also observed with rhizobial nodulation signals (Nod factors; lipo-chitooligosaccharides), which systemically suppressed nodule formation and AMF root colonization. These results support the hypothesis of a similar autoregulatory mechanism in both symbioses (Vierheilig and Piché 2002; Vierheilig 2004a).

It has been reported that plant hormones such as ethylene and and cytokinin are involved in the development of the rhizobial symbiosis (Penmetsa and Cook 1997; Lohar et al. 2004). In a previous study, Caba et al. (2000) investigated whether levels of the phytohormones cytokinin, indole-3-acetic (IAA) or abscisic acid (ABA) in supernodulating soybean mutants differ from those of wild-type soybeans. The obtained data suggested that cytokinin, IAA and abscisic acid ABA could be involved in the autoregulation of nodulation (Caba et al. 2000). However, no data are available on a possible role of jasmonic acid (JA) in the establishment of the rhizobial symbiosis. Studies with AMF report that roots from various host plants respond to AMF with various metabolic changes, including alterations of phytohormone levels, e.g. of IAA, ABA and JA (Ludwig-Müller 2000; Torelli et al. 2000; Vierheilig and Piché 2002; Ferguson and Mathesius 2003). Thus, phytohormones have been proposed as "suitable candidates for signalling between plants and AMF" (Ludwig-Müller 2000) and it is tempting to speculate that phytohormones are also regulatory signalling compounds in the autoregulation of mycorrhization.

In the present work, we studied the mycorrhizal autoregulation in split-root systems of soybeans (cv. Bragg). We found that the supernodulating mutant *nts1007* lacks systemic suppression of mycorrhization. We also determined the root contents of IAA, ABA and JA and discuss their potential role in autoregulation of mycorrhization.

Materials and methods

Biological material, growth conditions and experimental set-up

Seeds of wild-type soybean (*G. max* L.) cv. Bragg and its supernodulating mutant *nts1007* (seeds were kindly provided by ARC Centre of Excellence for Integrative Legume Research, University of Queensland, Brisbane Qld 4072, Australia)(Carroll et al. 1985) were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water and germinated in pots in a steam-sterilized (40 min, 120°C) mixture of expanded clay and a commercial potting peat substrate (1:1, v:v).

Soybean plants were grown in a growth chamber (day/night cycle: 16 h; 23°C/8 h; 19°C; rel. humidity 50%).

After 7 days, tips of main roots were cut off to induce the development of lateral roots and plantlets were replaced into the same substrate described above. After another 8 days, plants were transferred into a split-root system recently described (Vierheilig et al. 2000a). The substrate (steam-sterilized at 40 min, 120°C) used in the split-root system consisted of a mixture of silicate sand, expanded clay and soil (1:1:1; by vol.).

Soybean plants were fertilized three times a week with 4 ml per side of split-root with a KNO_3 (0.808 g/l), $Ca(NO_3)_2$ 4 H_2O (1.808 g/l) solution. The split-root compartment system (for details see Vierheilig et al. 2000a) consists of two compartments containing the two halves of the soybean root system. The two compartments are separated on the side joining each other by an impermeable PVC membrane in order to prevent any flow or growth from one to the other side. Thus one side of the split root system can be inoculated with an AMF, while the other side remains non-inoculated. The two outer sides of the split-root compartment unit (consisting of two compartments) are equipped with nylon screens (60 µm mesh), which can be penetrated by hyphae but not by roots.

All experiments and determinations were repeated twice with 5 replicates per treatment. All values are given as means with SD.

Inoculation with AMF fungi

After the transfer of plants into a split-root system, one half of the split-root system was immediately inoculated with AMF (or left non-inoculated). To inoculate the first half of the split-root system (first inoculation), one side (nylon) of the split-root compartment unit was joined with an inoculum compartment equipped on one side with a nylon screen (60 µm mesh) (for details see Vierheilig et al. 2000a). The inoculum compartment contained beans (*Phaseolus vulgaris* L. cv. Sun Gold) colonized by *Glomus mosseae* (Nicolson and Gerdemann) Gerd. and Trappe (BEG 12; La Banque Européenne des Glomales; International Institute of Biotechnology; Kent; GB). After 15 days, root AM colonization reached 69 ± 4% in the wild-type Bragg and 61 ± 15% in the *nts1007* mutant. At this time point, all plants were inoculated with the same fungus by joining the second side of the split-root system with another AMF inoculum compartment (second inoculation).

Eleven days after the second inoculation, plants were harvested, roots were carefully rinsed with water and the root fresh weight was determined. Root aliquots were separated for the determination of root colonization. Roots for hormone analysis were immediately frozen at −20°C and thereafter freeze dried.

Determination of AMF root colonization

To estimate AMF root colonization, roots were cleared by boiling in 10% KOH and stained according to the method of Vierheilig et al. (1998) by boiling in a 5% ink (Shaeffer; black)/usual household vinegar (= 5% acetic acid) solution. Stained roots were observed with a light microscope to determine the percentage of root colonization according to a modified method of Newman (1966).

Determination of IAA, ABA and JA

To investigate potential changes in phytohormone levels related to autoregulation of mycorrhization, IAA, ABA and JA levels were determined in non-inoculated roots and split-root systems. In split-root systems one side was mycorrhizal (15 days after the inoculation of one side of the split-root system) and the other side non-mycorrhizal.

Freeze dried roots (minimum 0.1 g fresh weight per individual analysis) were extracted with a mixture of isopropanol and acetic acid (95:5, v/v). To each sample 100 ng $^{13}C_6$-IAA (Cambridge Isotope Laboratories, Andover, MS, USA), 100 ng (d_6)-ABA (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada) and 50 ng (d_6)-JA (Miersch 1991) were added. Sample preparation was performed according to Walker-Simmons et al. (2000) with minor modifications. The samples were incubated under continuous shaking (500 rpm) for 2 h at 4°C. The samples were then centrifuged for 10 min at 10,000 *g*, the supernatant removed and evaporated to dryness under a stream of N_2. The residue was resuspended in methanol, centrifuged again for 10 min at 10,000 *g*, the supernatant removed and placed in a glass vial. The methanol was evaporated under a stream of N_2 and the sample was resuspended in 100 µl ethyl acetate. Methylation was carried out according to Cohen (1984) with freshly prepared diazomethane. For GC-MS analysis the sample was resuspended in ethyl acetate.

The GC-MS analysis was carried out on a Varian Saturn 2100 ion-trap mass spectrometer using electron impact ionization at 70 eV, connected to a Varian CP-3900 gas chromatograph equipped with a CP-8400 autosampler (Varian, Walnut Creek, CA, USA). For the analysis 2.5 µl of the methylated sample dissolved in 20 µl ethyl acetate was injected in the splitless mode (splitter opening 1:100 after 1 min) onto a Phenomenex ZB-5 column, 30 m×0.25 mm×0.25 µm using He carrier gas at 1 ml min^{-1}. Injector temperature was 250°C and the temperature program was 60°C for 1 min, followed by an increase of 25°C min^{-1} to 180°C, 5°C min^{-1} to 250°C, 25°C min^{-1} to 280°C, then 5 min isothermically at 280°C. The methyl esters of JA, IAA and ABA eluted under these conditions at 9.7, 13.5 and 16.2 min, respectively. Transfer line temperature was 280°C. For higher sensitivity, the µSIS mode (Varian Manual; Wells and Huston 1995) was used. Scan rate was 0.6 s $scan^{-1}$, multiplier offset voltage 200 V, emission current 30 µA and the trap temperature 200°C.

The endogenous hormone concentrations were calculated by the principles of isotope dilution (Cohen et al. 1987). For the determination of IAA, the molecular and quinolinium ions of the methylated substance at *m*/*z* 189/195 and 130/136, respectively, were monitored (ions deriving from endogenous and $^{13}C_6$-IAA; Cohen et al. 1986) and the calculation was done on the basis of the quinolinium ions. For the determination of JA, the ions of the methylated substance at m/z 224/230 and 151/155, respectively, were monitored (ions deriving from endogenous and d_6-JA; Creelman et al. 1992) and the calculation was done on the basis of *m*/*z* 224/230. For the determination of ABA, the ions of the methylated substance at *m*/*z* 190/194 and 162/166, respectively, were monitored (ions deriving from endogenous and d_6-ABA; Walker-Simmons et al. 2000) and the calculation was done on the basis of m/z 190/194.

Results

Effect of prior AMF on subsequent AMF colonization

Split-root systems are a useful tool to study autoregulation, i.e. systemic suppression of subsequent root colonization by an already colonized part of the root. We used wild-type soybean cv. Bragg and its supernodulating *nts1007* mutant, which is deficient in rhizobial

autoregulation (Caetano-Anollés and Gresshoff 1991; Carroll et al. 1985; Searle et al. 2003). After cutting off the tip of the main root, plants developed lateral roots within a relatively short time and formed a homogenous root system, which was separated into two equal parts.

We investigated whether pre-inoculation of one side of the root system of wild-type or mutant plants influences subsequent AMF colonization on the other part. The first half of the split-root system was inoculated with the mycorrhizal fungus *G. mosseae*. Control wild-type or mutant plants were not inoculated on this side of the split-root system.

After 15 days, all plants were inoculated with *G. mosseae* on the second half of the split-root system. 11 days later, AMF colonization was investigated on harvested roots (for details see Materials and methods). The obtained data clearly showed that in the wild-type Bragg, the degree of AMF colonization in the second side of the split-root system depended on the treatment of the first side (Fig. 1). Compared to control plants without pre-inoculation (43 ± 9%), subsequent AMF colonization was reduced in plants already colonized by *G. mosseae* (22 ± 3%). However, a different pattern of AMF root colonization could be observed in the *nts1007* mutant. Pre-inoculation of the first side of the split-root system did not affect further root colonization on the second side (without pre-inoculation 32 ± 5%, with pre-inoculation 31 ± 8%). These data indicate that mycorrhization is not autoregulated in the *nts1007* mutant.

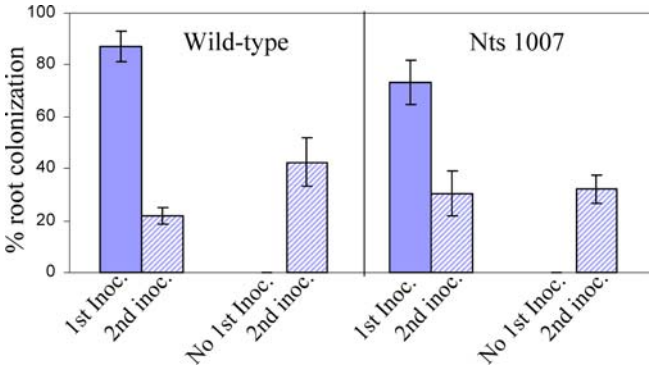

Fig. 1 Effect of prior mycorrhization (first inoculation) of one side of a split-root systems of the wild-type soybean and the supernodulating soybean mutant *nts* 1007 on subsequent mycorrhization (second inoculation) of the second side of a split-root system (means ± SD; $n = 5$)

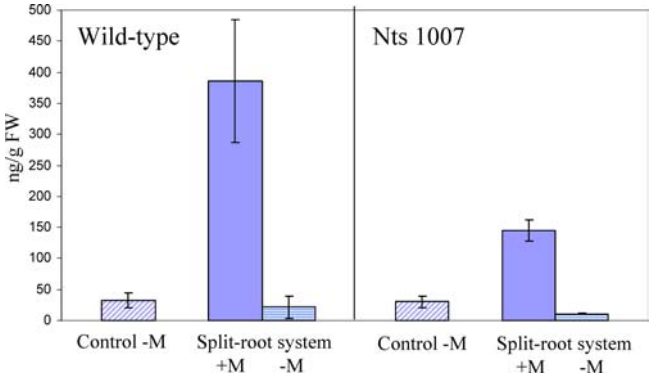

Fig. 2 IAA content in non-mycorrhizal (control −M) soybeans (wildtype and supernodulating *nts* 1007 mutant) and in the split-root system of mycorrhizal soybeans with one side mycorrhizal (+M) and the other side non-mycorrhizal (−M) (means ± SD; $n = 5$)

Effect of mycorrhization on IAA, ABA and JA

Autoregulation of nodulation has been reported to result in alterations of IAA and ABA levels in soybean roots (Caba et al. 2000). This prompted us to measure potential changes of IAA and ABA in mycorrhizal split-roots. We included JA in our study, as JA is a possible candidate to regulate the degree of mycorrhizal colonization. Phytohormone levels of wild-type and mutant soybeans were quantified in non-inoculated roots (see Figs. 2, 3; Control −M) and split-root systems. The obtained data indicated that IAA levels of wild-type Bragg and its *nts1007* mutant were similar in the absence of AMF fungi (Fig. 2). Mycorrhization resulted in both soybean lines in a clear accumulation of IAA. In the *nts1007* mutant however, the increase was considerably lower and reached only about half of that observed in mycorrhizal roots of wild-type plants. In the wild-type and the *nts1007* mutant, levels in the non-inoculated part of the AMF split-root system remained low and were similar to those of non-mycorrhizal control plants.

In non-inoculated roots, ABA levels were higher in wild-type plants than in the *nts1007* mutant (Fig. 3). Mycorrhization resulted in a strong increase of ABA and levels reached similar values in both lines. The ABA content in the non-inoculated part of the split-root system remained low. In wild-type plants, ABA levels were even lower than in non-mycorrhizal wild-type control plants. The *nts1007* mutant however, exhibited in the non-inoculated part of the AMF split-root system, higher ABA contents than in non-mycorrhizal control plants.

In non-inoculated split-root systems, levels of JA were higher in the *nts1007* mutant than in wild-type plants (Fig. 4). Mycorrhization resulted in a drastic increase of JA levels. Both lines accumulated similar amounts of JA in response to the AMF fungi. The wild-type Bragg and the *nts1007* mutant did not differ in their JA levels in the non-inoculated part of the AMF split-root system.

Discussion

Wild-type soybeans (cv. Bragg) inoculated with AMF show a systemic regulatory mechanism that suppresses further mycorrhization. Prior AMF root colonization on one side of the split-root system resulted in a clear reduction of root colonization on the other side. These

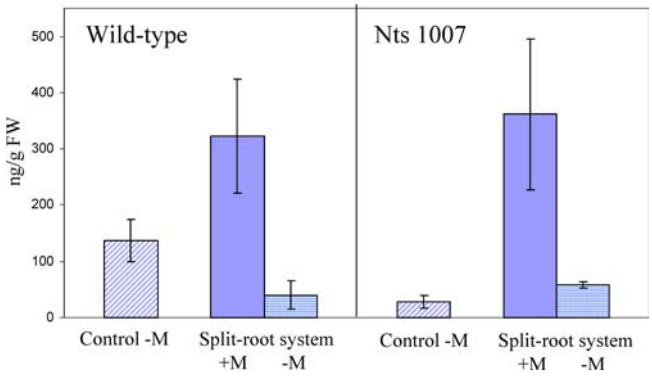

Fig. 3 ABA content in non-mycorrhizal (control −M) soybeans (wildtype and supernodulating *nts* 1007 mutant) and in the split-root system of mycorrhizal soybeans with one side mycorrhizal (+M) and the other side non-mycorrhizal (−M) (means ± SD; $n = 5$)

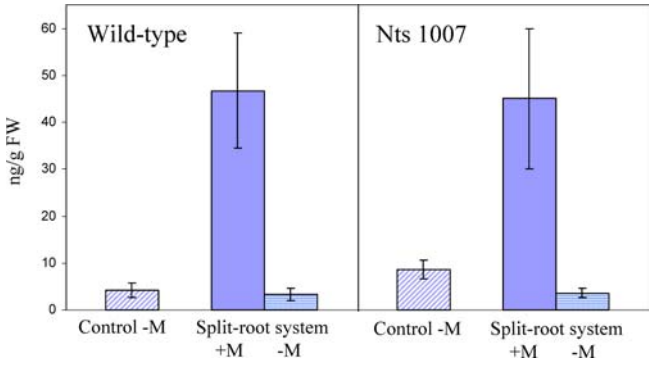

Fig. 4 The JA content in non-mycorrhizal (control −M) soybeans (wildtype and supernodulating nts 1007 mutant) and in the split-root system of mycorrhizal soybeans with one side mycorrhizal (+M) and the other side non-mycorrhizal (−M) (means ± SD; $n = 5$)

data show that the autoregulation of mycorrhization is not limited to barley and alfalfa (Vierheilig et al. 2000a, b; Catford et al. 2003), but seems to be general mechanism in host plants of AMF. These data are in agreement with previous reports demonstrating that supernodulating mutants of soybean (Shrihari et al. 2000), *L. japonicus* (Solaiman et al. 2000) and *Pisum sativum* (Morandi et al. 2000) exhibited an increase in arbuscule formation in comparison with their corresponding wild-type plants. Moreover, a recent study indicated that the *L. japonicus* hypernodulation mutant *har1* is hyperinfected with the nematode *Meloidogyne incognata* (Lohar et al. 2003), suggesting that CLAVATA1-like receptor kinases from legumes also play a role in plant–pathogen interactions.

In our study with soybean, we used *nts1007*, a well-characterized Bragg mutant of soybean, which is mutated in the receptor kinase gene *GmNARK* (Searle et al. 2003). Compared with wild-type plants, roots from the *nts1007* mutant responded to a first AMF inoculation with a similar frequency of AMF root colonization. These data are in agreement with the AMF colonization data of the Bragg wild-type and the *nts1007* mutant obtained by Wyss et al. (1990). In our split-root systems however, the *nts1007* mutant did not show an inhibitory effect of prior AMF colonization on further root colonization. In other words, fungal pre-colonization of one side of the root system did not systemically suppress mycorrhization on the other side. Similar autoregulatory feedback mechanisms of nodulation and mycorrhization have been suggested earlier (Vierheilig and Piché 2002; Vierheilig 2004a). Our observations seem to confirm this hypothesis and indicate that mutation of the *GmNARK* gene results not only in a supernodulating phenotype, but also abolishes mycorrhizal autoregulation.

Caba et al. (2000) measured several phytohormone levels in Bragg and the *nts1007* mutant before and after rhizobial inoculation with *Bradyrhizobium japonicum*. The authors suggested that phytohormones are involved in the rhizobial autoregulation of soybeans. The first key component of the legume autoregulation system however, the CLAVATA1-like receptor kinases (Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2003), did not provide an indication of the way phytohormones could be part of this control mechanism. Our data suggest that mycorrhizal colonization is associated with phytohormonal changes and it is tempting to speculate that phytohormones are also regulatory factors of mycorrhizal autoregulation.

When inoculated with *B. japonicum*, wild-type Bragg and the *nts1007* mutant accumulated similar levels of IAA (Caba et al. 2000). We found an IAA induction in mycorrhizal roots, indicating a general IAA response in both symbioses. However, whereas the degree of IAA induction was similar in rhizobial roots of Bragg and its mutant (Caba et al. 2000), IAA levels in our study increased more in mycorrhizal roots of Bragg compared to those of the *nts1007* mutant. These findings suggest that a functional GmNARK receptor kinase is required for maximal induction of IAA in mycorrhizal soybean roots.

In earlier studies with maize and tobacco, no effect of mycorrhization on the IAA levels has been detected (Danneberg et al. 1992; Kaldorf and Ludwig-Müller 2000; Ludwig-Müller et al. 1997; Shaul-Keinan et al. 2002). Recently, Torelli et al. (2000) found in mycorrhizal onion plants an IAA increase, similarily to that observed in the soybean plants used in our study. Interestingly, in onions with a high P status, IAA levels were elevated to a similar extent as in mycorrhizal onions (Torelli et al. 2000). As high P levels in plants negatively affect root colonization by AMF (Smith and Read 1997), a high IAA accumulation in high P plants could indicate an involvement of IAA in the reduction of AMF root colonization. This is consistent with the findings that treatment of roots with auxin transport inhibitors increased AMF colonization (Xie et al. 1998). Moreover, it has been reported that auxins strongly inhibit mycelium growth, sporulation and spore germination of *Fusarium culmorum* in vitro (Michniewicz and

Rozej 1987) and exogeneous application reduces *Botrytis* blight (Elad 1995). Auxins can also affect growth of AM fungi directly. IAA at micromolar concentrations had a strong inhibitory effect on the percentage of root segments containing proliferating intraradical hyphae of *G. fistulosum* (Gryndler et al. 1998).

In non-inoculated roots, Caba et al. (2000) found higher ABA levels in wild-type Bragg than in its *nts1007* mutant. Our non-inoculated roots confirm this difference in ABA levels. Interestingly, mycorrhization resulted in a strong ABA accumulation in Bragg and its *nts1007* mutant. Infection with *B. japonicum* did not significantly increase the ABA levels of soybean (Caba et al. 2000). These data indicate a clear difference between the two symbioses. In mycorrhizal maize an increase of ABA levels has been reported earlier (Danneberg et al. 1992). Interestingly this increase could be detected not only in the mycorrhizal root but also in the shoot, pointing towards a systemic accumulation of ABA. In our split-root experiments however, ABA levels of non-inoculated Bragg roots were similar to those of the *nts1007* mutant. An involvement of ABA in autoregulation of mycorrhization is therefore unlikely.

Jasmonic acid and its methyl ester have been found in a large number of plant species. They are known to regulate a number of physiological processes in plants and play a role in interplant signalling and in signal transduction in relation to defense gene induction (Dong 1998). The role of JA during the formation of the AM association remains unclear (reviewed by Ludwig-Müller 2000; Ferguson and Mathesius 2003; Vierheilig 2004a). Exogenous application of JA to papaya and cucumber results in a drastic decrease of AM root colonization (Ludwig-Müller et al. 2002). Moreover, it has been reported that in mycorrhizal barley and cucumber plants, JA levels are increased compared to non-mycorrhizal control plants (Hause et al. 2002; Vierheilig and Piché 2002). Until now, no data have been available on JA levels in supernodulating mutants. In our experiments, mycorrhization strongly induced the accumulation of JA, but the levels were similar in Bragg and its *nts1007* mutant. Both lines also showed similar JA levels in the non-inoculated side of AMF split-root systems. These data seem to exclude a direct involvement of JA in the mycorrhizal autoregulation.

To summarize, we found that soybeans with a mutated *GmNARK* gene are deficient in autoregulation of mycorrhization. This indicates that the autoregulation in the rhizobial and the mycorrhizal symbiosis involves the same receptor kinase in the signal transduction pathway. Split-root experiments with AMF showed that IAA levels might play a role in autoregulation of mycorrhization. Mycorrhizal colonization of soybeans also resulted in a strong induction of ABA and JA levels, but a role of these two phytohormones in mycorrhizal autoregulation is questionable.

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