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Two defined alleles of the LRR receptor kinase *GmNARK* in supernodulating soybean govern differing autoregulation of mycorrhization

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Plants regulate the extent of nodulation and root colonization by arbuscular mycorrhizal fungi (AMF), a phenomenon named autoregulation of symbiosis. We tested AMF colonization in split roots of various soybean genotypes [*Glycine max* (L.) Merr. cv. Bragg, Enrei, Harosoy and Williams], where precolonization of one side of the split-root system by the AMF *Glomus mosseae* resulted in reduced mycorrhization of the other. AMF precolonization failed to control secondary mycorrhization in the supernodulating Bragg nonsense mutant *nts1007* (Q106*), indicating that the *GmNARK* gene (predicted to encode a leucine-rich repeats (LRR) receptor kinase related to CLAVATA1 in *Arabidopsis*) is involved in autoregulation of the AMF symbiosis. Here, we tested whether the allelic *En6500* nonsense supernodulating mutant (*GmNARK* K606*, derived from cv. Enrei) and supernodulating mutants of cv. Williams (*Nod1-3* and *Nod2-4*) with yet-undefined genetic lesions exhibit a similar symbiotic phenotype in mycorrhizal split-root systems. Surprisingly, these supernodulating mutants retained their ability to autoregulate AMF. To examine possible differences between two allelic mutants, we determined levels of IAA, abscisic acid, coumestrol, daidzein and genistein in mycorrhizal and uninoculated control roots. Compared with wild-type plants, both mutants showed reduced IAA accumulation in mycorrhizal roots. Roots of cv. Enrei and *En6500* exhibited high levels of isoflavonoids not seen in Bragg or *nts1007*. Taken together, these findings showed that supernodulation mutants, despite a common nodulation phenotype, differ in their ability to autoregulate AMF root colonization. This suggests either that the *GmNARK* gene product of some mutants is still partially functional (Q106* vs. K606*) or that varietal differences reflected in altered physiological responses suppress the loss of function.

Abbreviations – ABA, abscisic acid; AM, arbuscular mycorrhizal; AMF, arbuscular mycorrhizal fungus; AOM, autoregulation of mycorrhization; AON, autoregulatory mechanism of nodulation; FW, fresh weight; HPLC, high-performance liquid chromatography.

Introduction

Legumes noticeably establish symbiotic associations with two soil-borne microorganisms, namely arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing rhizobia, to benefit their ability to sequester nitrogen and phosphorus resources. Increasingly data are accumulating that indicate similarities between signaling steps in the rhizobial and the AM symbioses (Endre et al. 2002, Guinel and Geil 2002, Hirsch and Kapulnik 1998, Stracke et al. 2002, Vierheilig 2004a, Vierheilig and Piché 2002), now suggesting 'evolutionary hijacking' of gene systems.

A systemically acting autoregulatory mechanism of nodulation (AON; Men et al. 2002) is known from the rhizobial interaction with legumes, as prior inoculation of one side of a split-root system suppresses nodule formation in the other root portion (Kosslak and Bohlool 1984, Olsson et al. 1989). This mechanism involves the perception of the *Rhizobium*-derived Nod factor, early stages of signal transduction leading to an 'activated state', which results in (1) initiation of cell division processes, (2) initiation of infection processes and (3) activation of the AON circuit (Fig. 1). Present understanding suggests that root tissue in the activated state signals leaf tissue by a yet-undefined molecule, possibly related to systemically acquired resistance mechanisms involved in pathogen perception. Within the leaf, an AON-controlling LRR receptor kinases called GmNARK in *Glycine max* (soybean; Searle et al. 2003), LjHAR in *Lotus japonicus* (Krusell et al. 2002, Nishimura et al. 2002) and MtSUNN in *Medicago truncatula* (Schnabel et al. 2005) respond directly or indirectly to the root signal and initiates a shoot-derived inhibitor, which, when translocated to the root, blocks further symbiotic development (Gresshoff 2003). Concurrently, changes in auxin translocation occur (van Noorden et al. 2006). Mutations in the *GmNARK* gene generally lead to increased nodule number, altered root growth [strongest in the *Ljhar1-1* mutant; weak in soybean possibly because of compensation by the related *GmCLV1A* gene (Searle et al. 2003)] and increased mycorrhizal colonization.

In parallel to AON (for details see Caetano-Anollés and Gresshoff 1991, Kinkema et al. 2006), autoregulatory events have also been suggested for the AM symbiosis (Vierheilig 2004a, Vierheilig and Piché 2002), consistent with the fact that mycorrhizal signaling involves several shared genes in the early stages of symbiosis signal perception [e.g., *SYMRK/NORK*, *DMI3* and *DMI1* but not the Nod factor receptor complex (Indrasunumar et al. 2007, Limpens et al. 2003, Radutoiu et al. 2003) encoded by *NFR1* and *NFR5*].

Evidence exists that deficiencies in the AON-LRR receptor kinase alter root growth (Kinkema et al. 2006). The *Ljhar1-1* mutant has reduced primary root growth, which is most pronounced in the *Rhizobium*-inoculated condition (Buzas and Gresshoff 2007, Wopereis et al. 2000). A somewhat milder restriction of root growth occurs in inoculated soybean supernodulator *nts382* (Day et al. 1986; allelic with *nts1007*). Reciprocal grafting of uninoculated root and shoot portions of *L. japonicus* showed that both root and shoot contribute to the cessation of root growth, while the hypernodulation phenotype is predominantly controlled by the shoot (Buzas and Gresshoff 2006, Jiang and Gresshoff 2002, Krusell et al. 2002, Nishimura et al. 2002). It is thus possible that *LjHAR1/GmNARK/MtSUNN* possess a non-symbiotic phenotype involved in root growth and a symbiotic one affecting AON. Both may rely on similar alterations in repressor molecules.

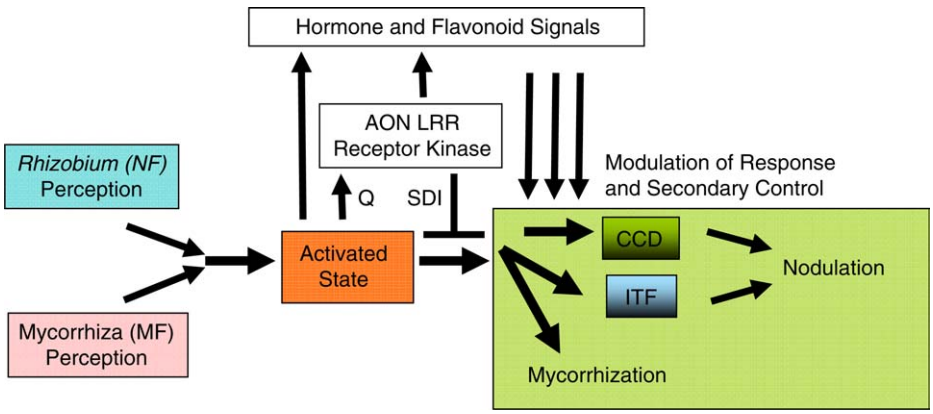

Fig. 1. Regulatory circuits linking *Rhizobium* and mycorrhizal symbioses. CCD is cortical cell divisions; ITF, infection thread formation; SDI, shoot-derived inhibitor; Q, root-derived autoregulation sensing cue; NF, nodulation factor (a decorate lipo-chito-*N*-acetylglucosamine oligomer; MF, the yet defined mycorrhizal factor (may be a cell wall component; may interact directly with SYMRK/NORK. Plant hormone responses may function in parallel to generate the 'autoregulated state'.

Autoregulation of root colonization by AMF is best studied in split-root systems, allowing separation of spatial and temporal events. Precolonization of one side of the split root resulted in a clear reduction of root colonization on the other side (Vierheilig et al. 2000a, 2000b). This suppression of secondary root colonization was linked with the intensity of mycorrhization on the first side (Vierheilig 2004b). Studying possible autoregulatory events between AMF and rhizobia, Catford et al. (2003) showed that nodulation on one side of a split-root system resulted in suppression of mycorrhization on the other side and *vice versa*. Furthermore, suppression of nodulation and mycorrhization were linked to an altered isoflavonoid accumulation pattern (Catford et al. 2006).

Soybean mutants *nts1007* (in cv. Bragg; mutated in Q106*) and *En6500* (in cv. Enrei; mutated in K606*) display an extreme supernodulation phenotype (Carroll et al. 1985). Both mutants carry nonsense mutations that truncate GmNARK, indicating that the receptor kinase is an essential component of the signaling circuit for AON (Nishimura et al. 2002, Searle et al. 2003). Mycorrhizal tests in a split-root system showed that the *nts1007* mutant is deficient in autoregulation of mycorrhization [the autoregulation of mycorrhization (AOM) minus phenotype]; an involvement of IAA in the signaling mechanism has been proposed (Meixner et al. 2005, van Noorden et al. 2006).

Testing AMF root colonization in *M. truncatula*, *P. sativum* (Morandi et al. 2000), *L. japonicus* (Solaiman et al. 2000) and soybean (Shrihari et al. 2000), various supernodulating mutants displayed an increased formation of arbuscules. From these data and our previous study (Meixner et al. 2005), we hypothesized that the lack of autoregulatory events could be linked with an enhanced formation of arbuscules.

Here we used a mycorrhizal split-root system to examine AOM in several supernodulating mutants (including two fully characterized alleles) of soybean to show that in contrast to *nts1007*, the *En6500* mutant and other supernodulating soybean mutants retained their ability to autoregulate the extent of AMF root colonization. Key metabolites [IAA, abscisic acid (ABA), coumestrol, daidzein and genistein], possibly acting downstream of GmNARK, were profiled in mycorrhizal and non-mycorrhizal roots to reveal that varietal differences may lead to the suppression of the effects of *GmNARK* mutant alleles.

Materials and methods

Biological material, growth conditions and experimental setup

Seeds of soybean (*G. max* L.) cv. Bragg and its supernodulating mutant *nts1007* (Carroll et al. 1985) were obtained from the ARC Centre of Excellence for Integrative Legume Research (University of Queensland, Brisbane, Australia). Soybean cv. Enrei and its supernodulating mutant *En6500* (Akao and Kouch 1992) and soybean cv. Harosoy and its Nod^- mutant $Harosoy^-$ (Francisco and Akao 1993) were kindly provided by Dr M. Takahashi (Legume Cultivation and Physiology Laboratory, National Institute of Crop Science, Tsukuba, Ibaraki, Japan). Soybean cv. Williams and its supernodulating mutants *Nod1-3* and *Nod2-4* (Gremaud and Harper 1989) were kindly provided by Dr J. C. Nicholas (USDA/ARS/Urbana Location, University of Illinois, Urbana, IL).

Seeds 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 cultivated in a growth chamber (day/night cycle: 16 h at 23°C and 8 h at 19°C; relative humidity 50%; minimal photoactive radiation 400 $\mu E\ s^{-1}\ m^{-2}$ by Radium HRI-T4W/DH lamps). After 10 days, tips of main roots were removed to induce the development of lateral roots, and plantlets were replaced into the same substrate described above. After another 7 days, split-root systems were established as recently described (Vierheilig et al. 2000b). The substrate (steam sterilized for 40 min at 120°C) used for the split-root system consisted of a mixture of silicate sand, expanded clay and soil (1:1:1; v/v/v).

The split-root system consisted of two compartments containing the two halves of the soybean root system. The two compartments were separated by an impermeable polyvinylchloride membrane to prevent any flow or growth from one side to the other. This design allowed mycorrhizal inoculation of a split root on one side, while the other side remained uninoculated. The two outer sides of each split-root compartment were equipped with a Nylon screen (60-μm mesh), which can be penetrated by hyphae but not by roots. Plants were watered three times per week.

Inoculation with AM fungus

After the transfer of plants into a split-root system, one half was immediately inoculated with AMF (control plants were left uninoculated). To inoculate the first half of the split-root system (first inoculation), the outer side with the Nylon screen was joined with an inoculum compartment equipped on one side with an identical Nylon screen (for details see Vierheilig et al. 2000b). The inoculum compartment contained beans (*Phaseolus vulgaris* L. cv. Sun Gold) colonized by *Glomus mosseae* (Nicolson &

Gerdemann) Gerd. & Trappe (BEG 12; La Banque Européenne des Glomales, International Institute of Biotechnology, Kent, GB; http://www.kent.ac.uk/bio/beg/). After 17 days, root AM colonization reached 69 ± 3% in cv. Bragg, 40 ± 5% in *nts1007*, 60 ± 5% in cv. Enrei, 31 ± 3% in *En6500*, 38 ± 1% in cv. Williams, 50 ± 3% in the *Nod1-3* and 67 ± 8% in the mutant *Nod2-4*. We note that AM colonization of the different wild-types ranged from 38 to 69% and that the supernodulation mutants were either above or below the wild-type values.

At this time point, all soybeans were inoculated with the same fungus by joining the second side of the split-root system with another AMF inoculum compartment (second inoculation). Ten days after the second inoculation, plants were harvested, roots were carefully rinsed with water and root aliquots were separated for the determination of AMF root colonization.

Determination of AMF root colonization

To estimate AMF root colonization, roots were cleared by boiling in 10% KOH and stained by boiling in a 5% ink (Shaeffer; jet-black) or common household vinegar (=5% acetic acid; Vierheilig et al. 1998a, 2005). Stained roots were observed with a light microscope to determine the percentage of root colonization according to a modified method of Newman (1966).

Because limited amount of space and split-root compartment units, experiments with different soybean genotypes were performed at different times, and thus, the extent of AMF root colonization levels can only be compared between wild-type and mutant plants of any one particular genotype, and not between genotypes.

Plant material for phytohormone and isoflavonoid analysis

Levels of IAA, ABA, coumestrol, daidzein and genistein were determined in AMF-inoculated and uninoculated roots of cv. Bragg, *nts1007*, cv. Enrei and *En6500* (six plants per treatment).

Seeds were surface sterilized and then cultivated in a commercial potting peat substrate as described above. Plants (14 days old) were then transferred to 300-ml pots (one plant per pot) containing a mixture of autoclaved (121°C, 20 min) sand, expanded clay and soil (1:1:1; by volume). The plants were placed into a hole in the substrate where the inoculum (5 g per plant) had been previously added. The inoculum consisted of colonized root pieces of bean (*P. vulgaris* L. cv. Sun Gold), sporocarps, spores and hyphae of *G. mosseae* (BEG 12; see above) (Vierheilig et al. 1993).

Randomly distributed plants were grown in a greenhouse (day/night cycle: 16 h at, approximately, 23°C and 8 h at ca. 19°C, relative humidity 50–70%, minimal photoactive radiation 400 μE s^{-1} m^{-2} by Radium HRI-T4W/DH lamps) and watered three times per week.

After 7 weeks of plant growth in the pots, the plants were harvested and roots carefully rinsed with cold tap water. The fresh weight (FW) of roots was determined, and root aliquots were immediately frozen at −20°C and thereafter freeze-dried for later analysis of hormones and flavonoids. Aliquots of harvested roots were used to determine the degree of AMF root colonization as described above. Root colonization reached 69 ± 3% in cv. Bragg, 59 ± 7% in the *nts1007* mutant, 51 ± 3% in cv. Enrei and 44 ± 2% in the *En6500* mutant. Again it is noted that the supernodulators developed less AM colonization than their respective wild-types.

Determination of IAA and ABA

Aliquots of freeze-dried roots (minimum 0.1 g FW 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) and 100 ng (d_6)-ABA (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada) were added. Sample preparation and GC–MS analysis was performed as previously described in Meixner et al. (2005). For the determination of IAA, the quinolinium ions of the methylated substance at m/z 130/136 were monitored (ions deriving from endogenous and $^{13}C_6$-IAA; Cohen et al. 1986). For the determination of ABA, the ions of the methylated substance at m/z 190/194 were monitored (ions deriving from endogenous and d_6-ABA; Walker-Simmons et al. 2000). The endogenous hormone concentrations were calculated by the principles of isotope dilution (Cohen et al. 1986).

Determination of daidzein, genistein and coumestrol

Isoflavonoids from aliquots of freeze-dried roots (ca. 20 mg) were extracted with 1 ml methanol at room temperature during 24 h using a shaker at 150 rpm. The extracted solution was filtered through a filter paper, and thereafter, the solutions were centrifuged at 21 844 g for 5 min (at ambient temperature). The supernatant was further filtered through a 0.45-μm HV Durapore syringe-driven filter unit (Millipore Corporation, Billerica, MA) and samples (20 μl) were used for high-performance liquid chromatography (HPLC) analysis.

The Waters HPLC system was equipped with a 2487 Dual λ Detector, and 1525 Binary HPLC Pump,

controlled by the EMPOWER™ 2 software. A C18 5-μm 4.6 × 150-mm column (Symmetry) was installed, and the column temperature was kept with the TCM column-heater unit (Waters Corporation, Milford, MA) at 30°C. The mobile phase was a water/methanol gradient consisting of the following steps: (1) 0–10 min, linear gradient from 30 to 80% methanol; (2) 10–12 min, isocratic at 80% methanol; (3) 12–20 min, linear gradient from 80 to 100% methanol and (4) 20–25 min, isocratic at 100% methanol. The flow rate was 1 ml min^{-1}, and the column pressure varied between 14.5 ± 0.1 MPa (step 1) and 5.2 ± 0.1 MPa (step 4). Isoflavonoids were monitored at a wavelength of 254 nm. The peaks were quantified with the EMPOWER™ 2 analytical chromatography data system. The retention times of genistein, daidzein and coumestrol in standard solutions were 7.977, 8.142 and 9.859 min, respectively.

Analytical grade genistein (4′,5,7-trihydroxyisoflavone), daidzein (4′,7-dihydroxyisoflavone) coumestrol (7,12-dihydroxycoumestan) and methanol (HPLC grade) were purchased from Sigma Aldrich Chemical Co. (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) Double distilled water used for HPLC was further cleaned by a Millipore filter (0.45 μm). To prepare standard solutions, genistein, daidzein and coumestrol were dissolved in dimethyl sulfoxide (0.1 g ml^{-1}) and then 40× diluted with methanol.

Statistical analysis

Experiments were repeated twice with at least five replicates per treatment. Data of phytohormone and isoflavonoid measurements were statistically analyzed with the SPSS 11.0 software (SPSS Inc., Chicago, IL). Mean separation was carried out by using Tukey's multiple range test. Data in Table 1 were statistically analyzed by Fisher's least significant difference test.

Results

Inoculation of all tested soybean genotypes of the split-root system resulted in a high degree of AMF root colonization on the first side, ranging from 53 ± 5% in the *nts1007* mutant up to 87 ± 6% in the Bragg wild-type (Table 1). In all wild-type cultivars (Bragg, Enrei, Harosoy and Williams), precolonization on the first side of the split-root system induced a significant reduction of AMF root colonization on the other side. A similar systemic suppression was also seen in a nodulation mutant (Harosoy^{-}) that is unable to develop nodules. This mutant carries the *rj1* mutation, now shown to be a frameshift mutation leading to truncation of the GmNFR1α LysM-type receptor kinase, proposed to be part of the Nod factor receptor system (Indrasunumar et al. 2007). In *nts1007*, a supernodulating mutant of cv. Bragg, AMF precolonization on one side of the split-root system did not systemically suppress secondary mycorrhization (Table 1; Meixner et al. 2005). *En6500*, however, a supernodulating mutant of cv. Enrei and allelic to *nts1007*, retained its ability to systemically suppress mycorrhization as the degree of suppression by AMF precolonization was similar to that in wild-type plants. AMF precolonization in *Nod1-3* and *Nod2-4*, two supernodulating mutants of

Table 1. Effect of AMF precolonization on secondary AMF root colonization in soybean split roots. Split-root systems were established and then inoculated on one side with *G. mosseae* BEG 12 (with AMF on first side). Control plants were not inoculated (without AMF on first side). After 17 days, the first side of inoculated split roots was strongly colonized by AMF (the colonization degree varied between 53 and 87%, depending on the performed experiment). All plants were then inoculated with the same fungus on the second side of the split-root system. Data indicate the percentage of root colonization (mean values) in the second side of the split root at the time of harvest (10 days post-inoculation). Values of each plant genotype followed by the same letter are not significantly different according to Fisher's least significant difference test ($P < 0.05$). The degree of suppression by precolonization (expressed in %) was calculated as follows: [(x − y)/x] *100, where x = AMF root colonization (%) on the second side without AMF on the first side; y = AMF root colonization (%) on the second side with AMF on the first side. Abbreviations for nodulation phenotypes: Nod^{+}, nodulation; Nod^{++}, supernodulation; Nod^{-}, no nodulation.

Genotype	Nodulation phenotype	AMF root colonization on second side (%)		Degree of suppression by precolonization (%)
		Without AMF on first side	With AMF on first side	
cv. Bragg (wild-type)	Nod^{+}	42.5a	22.2b	48
nts1007 (mutant of Bragg)	Nod^{++}	14.6a	15.7a	−7
cv. Enrei (wild-type)	Nod^{+}	27.8a	13.3b	52
En6500 (mutant of Enrei)	Nod^{++}	19.5a	9.6b	51
cv. Harosoy (wild-type)	Nod^{+}	35.0a	22.8b	35
Harosoy^{-} (mutant of Harosoy)	Nod^{-}	35.2a	19.7b	44
cv. Williams (wild-type)	Nod^{+}	34.0a	18.6b	45
Nod1-3 (mutant of Williams)	Nod^{++}	32.7a	12.9b	61
Nod2-4 (mutant of Williams)	Nod^{++}	31.4a	10.5b	67

cv. Williams with yet-undefined molecular lesions, also resulted in a suppression of secondary AMF root colonization (Table 1).

As suggested previously (Meixner et al. 2005), phytohormones are likely to be involved in mycorrhizal autoregulation of soybean. To examine possible differences between *nts1007* and *En6500*, levels of IAA and ABA were determined in mycorrhizal roots and uninoculated control roots. Corresponding wild-type plants were included into the study. AMF colonization considerably increased IAA levels in all tested plants. Strongest stimulation was observed in roots of cv. Bragg. In *nts1007* and *En6500*, AMF colonization resulted in significant lower IAA levels, indicating that a functional *GmNARK* is required for maximal IAA accumulation in AMF colonized roots (Fig. 2A). ABA levels were similar in all tested uninoculated soybeans; however, a significant increase was observed in mycorrhizal roots of *nts1007* (Fig. 2B).

Isoflavonoids are other plant metabolites that modulate establishment of the AMF symbiosis (Vierheilig 1998b). We therefore tested whether *nts1007* and *En6500* differentially accumulate isoflavonoids. Coumestrol levels were highest in uninoculated and AMF colonized roots of *En6500* (Fig. 3A). In roots without mycorrhizae, daidzein levels in Enrei and its *En6500* mutant were much higher than in Bragg and *nts1007*. Mycorrhization reduced the daidzein levels in roots of Enrei and *En6500* (Fig. 3B). Similar to daidzein, genistein levels in uninoculated Enrei and *En6500* roots were higher than in Bragg and *nts1007*. Mycorrhization strongly reduced the levels of genistein in Enrei and *En6500* (Fig. 3C).

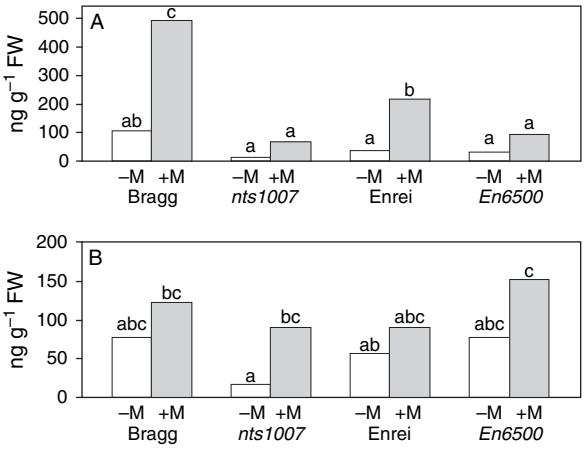

Fig. 2. Levels of free IAA (A) and ABA (B) in roots of non-mycorrhizal (control −M) and AMF colonized (+M) wild-type soybean plants (cv. Bragg and cv. Enrei) and their respective supernodulating mutants (*nts1007* and *En6500*). Values indicated with the same letter are not significantly different according to Tukey's multiple range test ($P < 0.05$).

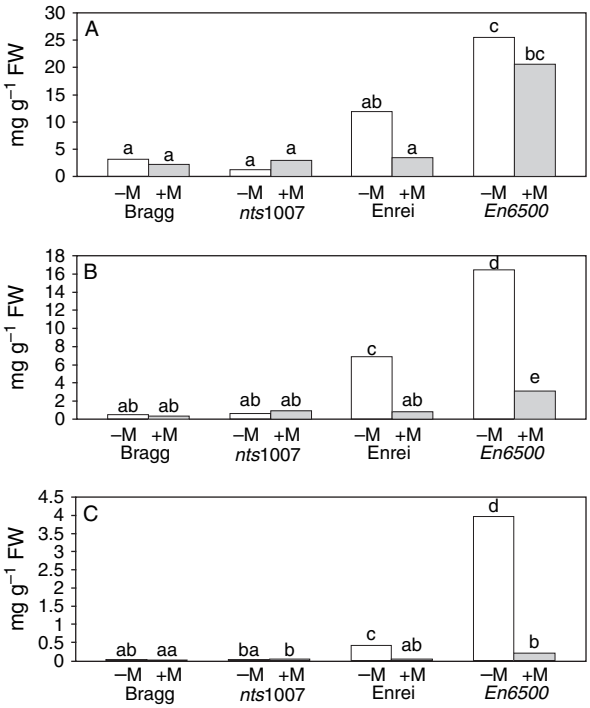

Fig. 3. Levels of coumestrol (A), daidzein (B) and genistein (C) in soybean roots. Isoflavonoid levels were determined in roots of non-mycorrhizal (control −M) and AMF colonized (+M) wild-type plants (cv. Bragg and cv. Enrei) and their respective supernodulating mutants (*nts1007* and *En6500*). Values indicated with the same letter are not significantly different according to Tukey's multiple range test ($P < 0.05$).

Discussion

In soybean, barley and alfalfa, AMF precolonization of one side of a split-root system resulted in a systemic suppression on the other side (Catford et al. 2003, 2006, Meixner et al. 2005, Vierheilig 2004b, Vierheilig et al. 2000a, 2000b). The soybean mutant *nts1007* has a nonsense mutation in *GmNARK* (Q106*) and is therefore unable to autoregulate nodulation (Searle et al. 2003) and mycorrhization (the AOM$^-$ phenotype; Meixner et al. 2005). The mutation is expected to result in a truncated protein, which if not degraded, would only represent the first three LRR domains and two N-terminal 'paired cysteines'. From these studies, it was concluded that AON and AOM are controlled in a similar manner and that GmNARK is an essential component of the signaling mechanism.

This work shows inconsistencies in this hypothesis and suggests further complexities, most likely based on metabolic interactions caused by varietal differences. Specifically, the supernodulating mutants *En6500*, *Nod1-3* and *Nod2-4* retained their ability to sense whether parts of the root system have been previously colonized by

AMF. In all these mutants, precolonization suppressed secondary mycorrhization, indicating that the supernodulation phenotype per se does not correlate with the AOM-minus phenotype.

The results from our test with the *EN6500* plants are of particular interest, as its mutation affects also the *GmNARK* gene (Searle et al. 2003). Our data indicate that *GmNARK* is essential for mycorrhizal autoregulation in cv. Bragg, whereas cv. Enrei has the ability to autoregulate AMF root colonization in the absence of a functional *GmNARK* gene. Formation of arbuscules has been described to be stimulated in mutants *En6500* and *Nod1-3* (Shrihari et al. 2000), an observation that we could only partially verify as *En6500* repeatedly showed reduced mycorrhization compared with its parent. Similar observations were also made for Bragg and *nts1007*. However, we also found that *Nod1-3* and *Nod2-4* had increased mycorrhization compared with the Williams parent. Apparently, inactivation of a *GmNARK*-related signaling pathway in *En6500* resulted in supernodulation and stimulation of arbuscule formation. Autoregulation of mycorrhization in our split-root systems was still functional in *En6500*, indicating a *GmNARK*-independent mechanism, or that the physiological status of Enrei is different than that of Bragg, leading to suppression of the *GmNARK*-deficient condition.

Cultivar differences were shown to exist for several key metabolite responses possibly connected pleiotropically to the action of *GmNARK*. For example, *GmNARK* may regulate processes related to jasmonic acid and ABA metabolism. Bragg and Enrei may have different response thresholds to regulation in this area. Thus, Enrei continues to provide a response, while Bragg does not.

Alternatively, the explanation may reside in the molecular nature of the mutation. *nts1007* is truncated early, and the resultant protein would contain as little as three LRR domains and a pair of cysteines. In contrast, *En6500* (K606*) is mutated close to the transmembrane domain and would possess two sets of paired cysteines (flanking both sides of the LRR portion) as well as the full LRR complement. These paired cysteines are presumed to be involved in inter- and intramolecular interactions. The central region of the LRR portion may contain an 'island region' itself proposed to be involved in intermolecular coupling. Thus, truncated proteins of that nature may retain the ability to bind or compete with their normal interactors (cf., CLAVATA2 protein appears to be a truncated version of CLAVATA1 and still binds in a functional complex; Fletcher 2002). Thus, *En6500* may retain sufficient activity to facilitate AOM but not AON.

Recent analyses of the supernodulation phenotype and/or the AOM-minus phenotype have focused on finding links with phytohormone levels in the plant (Ferguson and Mathesius 2003, van Noorden et al. 2006, Wopereis et al. 2000). Caba et al. (2000) suggested that IAA and ABA could be involved in the autoregulation of nodulation, and Meixner et al. (2005) suggested an involvement of IAA in the mycorrhizal autoregulation. van Noorden et al. (2006), studying the *MtSUNN* mutant altered in a *GmNARK* orthologue in *M. truncatula*, found that the mutant translocated increased amounts of IAA from the shoot independent of its inoculation status. Such findings contrast with our present observation that auxin levels of *GmNARK* mutants are reduced.

Our results provide additional evidence that *GmNARK*-dependent signaling events stimulate IAA levels in mycorrhizal roots. The obtained data, however, cannot explain the differences in mycorrhizal autoregulation between *nts1007* and *En6500*. Similarly, the measured ABA levels indicate accumulation of ABA in AMF colonized roots but do not point to a significant difference between the two mutants.

The involvement of flavonoids as signaling compounds in the AM symbiosis has been proposed in a number of studies (reviewed by Morandi 1996, Vierheilig et al. 1998b). Changes in the flavonoid levels in roots of various plants through mycorrhization have been reported (e.g., Harrison and Dixon 1993, Larose et al. 2002, Volpin et al. 1994, 1995), and in a recent study with split-root systems of alfalfa plants providing first evidence that isoflavonoid levels are systemically regulated, a role of isoflavonoids in the autoregulation of mycorrhization has been suggested (Catford et al. 2006).

Few data are available on isoflavonoids in the soybean–AMF interaction. Coumestrol and daidzein applied to soybean roots (cv. Bragg) strongly stimulated AMF root colonization (Xie et al. 1995), suggesting that isoflavonoids from soybeans can regulate the extent of symbiosis. Morandi et al. (1984) showed that coumestrol and daidzein levels were not altered in response to colonization with *G. mosseae*. These findings are in agreement with our data for cv. Bragg and *nts1007*. However, with cv. Enrei and *En6500*, we saw a different accumulation pattern. All tested isoflavonoids were detected at higher levels, indicating cultivar-specific differences. Mycorrhization reduced the levels of isoflavonoids in Enrei and *En6500* (Fig. 3).

Because the supernodulating mutant *En6500* with high isoflavonoid levels showed a similar mycorrhizal autoregulation as the wild-type cv. Bragg with much lower isoflavonoid levels (both AOM^+ phenotype), and the supernodulating mutant *nts1007* with similar low isoflavonoid levels as the wild-type cv. Bragg was unable to autoregulate mycorrhization (AOM^- phenotype), it seems unlikely that isoflavonoid levels are involved in the expression of the AOM^- phenotype.

Further comparative studies with supernodulating AOM^+ and AOM^- phenotype mutants are needed to elucidate the mechanisms involved in the mycorrhizal autoregulation.

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