Alteration of plant growth and development by 
*Rhizobium nodA* and *nodB* genes involved in the 
synthesis of oligosaccharide signal molecules

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Summary

The highly conserved *Rhizobium* nodulation genes 
nodABC are required to produce lipid-linked chito-
oligosaccharide signal molecules which elicit nodule 
organogenesis in roots of leguminous plants. 
Recently, it has been shown that NodB deacetylases 
chitoooligosaccharides at the non-reducing terminus, 
so that the free amino group of the chitoooligosaccha-
ride backbone can then be acylated by a specific fatty 
acid. The *Rhizobium* NodA protein together with the 
nodB encoded chitoooligosaccharide deacetylase are 
involved in generating small, heat-stable compounds 
that stimulate mitosis in protoplasts derived from 
either legumes or other plant species. To test 
whether these gene products could play a role in reg-
ulation of plant development, we introduced and 
expressed the *Rhizobium melliloti* nodA and nodB 
genes singly or in combination under the control of 
diverse promoters in tobacco. Altered phenotypes 
correlating with nodA and nodB gene expression in 
transgenic plants indicate that tobacco contains the 
necessary substrates for the NodA and NodB pro-
teins to produce signal molecules modulating plant 
growth and organ development.

Introduction

Rhizobia elicit nodule organogenesis in roots of legumi-
nous plants by secreting host-specific lipo-oligosaccha-
ride signal molecules (Truchet et al., 1991). These 
extracellular bacterial signals consist of an oligosaccha-
ride backbone of four or five β 1,4-linked N-acetylglu-
cosamine residues which carries at the non-reducing end 
a long-chain unsaturated fatty acyl group (Lerouge et al., 
1990; Schultz et al., 1992; Spahn et al., 1991). It has 
been shown that only the highly conserved *Rhizobium* 
nodulation genes nodABC are required to produce the 
basic structure of these modified chitoooligosaccharide 
signal molecules (Spahn et al., 1991). Recently, we pro-
vided evidence, that the NodB gene product is an oligo-
saccharide-modifying enzyme which deacetylates the 
non-reducing N-acetylglucosamine residue of chito-
ooligosaccharides (John et al., 1993). Moreover, we have 
shown that the deacetylase NodB together with the 
cytosolic protein NodA are sufficient to produce small, 
heat-stable compounds that stimulate mitosis in various 
protoplasts derived from legumes and non-legumes 
(Schmidt et al., 1988).

The studies reported in this paper were initiated to 
determine whether or not the nodA and nodB gene 
products would be functional in plants and whether their 
activity would influence growth and development. We 
therefore introduced and expressed the *Rhizobium melliloti* 
nodA and nodB genes separately or in combina-
tion in tobacco, and we analyzed the effects on growth 
and development caused by these genes in uninfected 
transgenic plants. The resulting transgenic plants showed 
characteristic morphological abnormalities indicating that 
substrate molecules are likely to be present in tobacco 
that allow the nodA and nodB encoded proteins to 
synthesize growth-controlling factors and also signal 
transduction pathways to respond to the presence of 
these regulatory molecules.

Results

Expression of nodA and nodB genes in plants

The construction of plant expression vectors carrying the 
nodA and nodB genes under the control of the cauliflower 
mosaic virus (CaMV) 35S promoter or the 1',2' dual Tr 
promoter of mannopine synthase is outlined in Figure 1 
and in Experimental procedures. To express the 
*R. melliloti* nodA and nodB genes separately or in combi-
nation, these T-DNA based plant vectors were introduced 
into tobacco (*Nicotiana tabacum* cv. Havana SR1) by 
Agrobacterium-mediated leaf disc infection (Horsch et al., 
1985).

For each construction (Figure 1) 20 independent 
kanamycin-resistant transformants were regenerated

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from which four independent transgenic lines were selected for further studies. Southern blot analyses of DNA extracted from leaves of the R₀ plants and of their F₁ progeny confirmed that the nod genes were integrated into the plant genome (data not shown). Backcrosses with SR1 tobacco revealed that the nod genes in transgenic plants were inherited in a Mendelian fashion. Analysis of the F₁ progenies of transgenic tobacco revealed that in the pPCV701nodAB plants (Figure 1) the kanamycin-resistance marker was transmitted as a single Mendelian locus. In the case of pPCV702nodAB and pPCV702nodB (Figure 1), respectively, the test crosses showed integrations on one or two unlinked chromosomal loci. All subsequent studies of growth and development were performed with the F₁ generation of the kanamycin resistant transgenic plants containing the appropriate nod gene inserts.

Expression of the nodA and nodB genes in transgenic tobacco plants was analyzed by Northern hybridization (Figure 2) and immunoblotting (data not shown). Morphological alterations observed in the transgenic plants were shown to correlate with the presence of nod transcripts. The blots were probed with DNA fragments specific for the nodA and nodB genes, respectively. These specific probes hybridized to transcripts of approximately 0.7 kb which correlates with the expected size of mRNAs encoded by the inserted nod genes. No cross-hybridization was detected between the nod probes and mRNAs isolated from untransformed plants (Figure 2a, lanes 4 and 8). The Northern blot shows that the nodA and nodB mRNA levels were high when transcription was driven by the CaMV 35S promoter (lanes 1 and 6). Transcript analysis of flowering transgenic plants expressing nodA and nodB simultaneously under the control of the 1',2' dual T₅ promoter shows a strong hybridization signal for nodA (lane 3) but no detectable nodB-specific transcripts (lane 7). However, mRNA homologous to nodB was detected in poly(A)+ RNA isolated from different tissues of young nodAB transgenic plants (Figure 2b, lanes 7–12). The finding that nodB was expressed proportionally weaker than nodA was expected and explained by the fact that transcription of the nodB gene was driven by the 1' end of the dual T₅ promoter (see Figure 1) imparting a sevenfold weaker expression level relative to the corresponding 2' end of the promoter (Leung et al., 1991).

To express both nodA and nodB genes under the control of the strong CaMV 35S promoter, tobacco plants transgenic for nodA were crossed with nodB transgenic plants. Northern analysis of the offspring of these crosses showed that equal amounts of nodA- and nodB-specific transcripts had been produced in the corresponding transgenic plants (Figure 2c, lanes 3 and 7, 4 and 8).

Using antibodies directed against NodA and NodB (Schmidt et al., 1986, 1988) we verified the presence of the corresponding Nod proteins in the transgenic plant tissues by Western blot analysis (data not shown).

Simultaneous expression of nodA and nodB driven by the dual T₅ promoter leads to the formation of bifurcated leaves and stems

In most cases expression of nod genes in the transformed tissue did not interfere with plant regeneration. However, during regeneration of transgenic plants
expressing nodAB from the TR promoter, we observed an inhibition of root formation when shoots freshly cut from transformed leaf tissue were grown on hormone-free medium. After 6 weeks on hormone-free medium rooting was still inhibited and an increased number of shoots developed which partially grew into the agar (Figure 3a). To induce root formation shoots were excised again and were grown further on hormone-free medium. Examination of the F1 progeny of the regenerants revealed characteristic alterations during plant development. In pPCV701nodAB transgenic plants the formation of single bifurcated leaves was observed (Figure 3b). Probably due to the low activity of the phytohormone-regulated TR promoter (Langridge et al., 1989) we observed this local event only in the basal region of young plants where both nod genes were transcribed (for Northern data, see Figure 2b). Figure 3(c) shows that this alteration of leaf morphology was found not only in the middle or upper part of the midrib but also in the petiole so that well-separated leaves were obtained. This effect on organogenesis can also lead to the formation of two or more stems emerging independently of the leaf axile (Figure 3d). These phenotypic alterations described above were found in 50% of the pPCV701nodAB transgenic plants.

Figure 2. Identification of nodA and nodB mRNAs in transformed tobacco plants.

Transcription of the nod genes was under the control of the CaMV 35S promoter (p35S) or the 1.2' dual TR promoter (TR). Northern blots were hybridized as indicated to specific radiolabelled probes containing part of the nodA or nodB coding regions (see Experimental procedures).

(a) Analysis of poly(A)+ RNA extracted from lower leaves of flowering plants. Lanes 1 and 5, 5 μg RNA of pPCV702nodA transgenic plant; lanes 2 and 6, 5 μg RNA of pPCV 702nodB transformant; lanes 3 and 7, 10 μg RNA of pPCV701nodAB transgenic plant; lanes 4 and 8, 10 μg RNA of wild-type SR1 tobacco plant (control).

(b) Northern hybridization of poly(A)+ RNA (5 μg) extracted from 3–4-week-old pPCV701nodAB transgenic plants. Lanes 1–3 and 7–9, RNA from leaves (L), stems (S) and roots (R) of a nodAB transgenic plant. Lanes 4 and 10, 5 and 11, 6 and 12, respectively, RNA from leaves of three independent transformants.

(c) Analysis of poly(A)+ RNA (5 μg) extracted from leaves of crossed plants. Lanes 1 and 5, crossing of pPCV702nodA transgenic plant with wild-type SR1 tobacco (control); lanes 2 and 6, crossing of pPCV702nodB transgenic plant with the wild-type SR1 tobacco (control); lanes 3 and 7, 4 and 8, respectively, two independent crossings of pPCV702nodA with pPCV702nodB transgenic plants.

Separate expression of nodA and nodB cause distinct morphological alterations

Transgenic tobacco expressing nodA from the strong CaMV 35S promoter showed slightly reduced growth, a reduction in the internode distance, and an altered leaf morphology when compared with SR1 control plants (Figure 4a). With increasing distance from the apex the nodA transgenic plants develop rounded and wrinkled leaves (Figure 4a and d).

The most striking phenotype of the transgenic plants synthesizing the chitooligosaccharide deacetylase NodB was a significantly reduced rate of growth (Figure 4b). Moreover, the nodB transgenic plants exhibited an altered leaf morphology (Figure 4d) and a compact inflorescence (Figure 4c). Many flowers had only four petals and four anthers and the amount of pollen was reduced. In all cases we found heterostyly with increased stigma size which resulted in plants that were unable to self-pollinate.

The morphological changes described above were found in all of the pPCV702nodA and pPCV702nodB transgenic plants, respectively. In contrast to transgenic plants expressing nodA and nodB simultaneously from the dual TR promoter, no bifurcated leaves and stems were observed with transgenic plants expressing either nodA or nodB genes alone.
Figure 3. Transgenic tobacco plants expressing nodA and nodB genes under control of the dual Tn7 promoter.
(a) Development of shoots on hormone-free LS medium.
(b) Growth of the F1 progeny of transformant pPCV701nodAB. Note the formation of a bifurcated leaf.
(c) Comparison of different bifurcated leaves from independent lines of pPCV701nodAB transgenic plants.
(d) Formation of two stems emerging independently of the leaf axile.

Figure 4. Separate expression of nodA and nodB genes in transgenic tobacco.
(a) Comparison of a pPCV702nodA transgenic plant (right) with a wild-type SR1 plant (left).
(b) Comparison of two independent lines of pPCV702nodB transgenic plants (right) with a wild-type SR1 plant (left).
(c) Compact inflorescence of transformant pPCV702nodB. Note that many flowers have only four petals. In all cases we found heterostyly.
(d) Comparison of leaves from a wild-type SR1 plant with leaves from tobacco plants transgenic for nodA, nodB and nodAB as indicated.
Plants expressing nodA and nodB in combination under CaMV 35S promoter control show different biological effects

In order to obtain transgenic plants simultaneously expressing nodA and nodB at the same level throughout plant development, transgenic tobacco expressing the single nodA gene from the 35S promoter was crossed with plants expressing nodB from the same promoter. Southern blot analysis of the DNA isolated from the offspring of these crosses verified that 27 out of 100 crossed plants contained both nod genes (for Northern data, see Figure 2c).

In all cases a high expression level of nodA and nodB (both under CaMV 35S promoter control) resulted in plants with different features to those observed with transgenic tobacco plants in which both genes were under the control of the phytohormone-regulated TR promoter (Figure 3). In contrast to these plants no bifurcated leaves or stems were found in the crossed CaMV 35S–nodAB transgenic tobacco plants. Relative to wild-type SR1 control plants, the CaMV 35S–nodAB transgenic tobacco plants obtained by these crossings displayed reduced growth, low apical dominance, and altered leaf morphology (Figure 5a). After flowering the leaves became extremely wrinkled (Figure 5b). In all flowers we observed heterostyly with an increased size of the stigma (Figure 5c). Obviously, the biological effects caused by the high level expression of both nodA and nodB in transgenic tobacco can be explained as a combination of morphological alterations which have been already shown for the transgenics expressing the single nod genes (Figure 4).

A comparison of the seed capsules of all transgenic plants described in this paper shows that, in particular, the size of the capsules and the number of seeds of the nodB transgenic plants were significantly reduced (Figure 6). Interestingly, in all F1 transgenic plants described in this paper no obvious alteration of root morphology was observed. In comparison with the wild-type plants there
were no significant differences in root growth nor in number of lateral roots at different developmental stages. Furthermore, the abundance of root hairs was similar as well as the overall organization of tissue.

Discussion

Transgenic tobacco plants expressing the rhizobial nodA and nodB genes in various combinations under the control of different promoters were generated. Depending on the level of expression, the transgenic plants showed distinct phenotypic alterations, e.g., reduced growth, altered leaf morphologies, and heterostyly. In Rhizobium, the cytosolic nodA and nodB proteins (Schmidt et al., 1986, 1988) together with the nodC transmembrane protein (John et al., 1988) are essential for the production of lipochitooligosaccharide signals, which are released by the bacteria and act on leguminous plants as nodule-inducing morphogens (Spank et al., 1991; Truchet et al., 1991). The finding that Nod signal molecules are modified chitooligosaccharides supports the assumption that specific oligosaccharides, designated as oligosaccharins, can serve as regulatory molecules in plants affecting growth and development (Albersheim et al., 1983). Such oligosaccharide signals can be released from the plant cell wall by hydrolases, and are effective in very low concentrations (Eberhard et al., 1989; Marfà et al., 1991; McDougall and Fry, 1988; Tran Than Van et al., 1985).

We have shown that expression of the nodB gene encoding a chitooligosaccharide deacetylase significantly affects the growth and development of transgenic tobacco plants (Figure 4). The NodB protein of Rhizobium deacetylates the non-reducing N-acetylgalcosamine residue of chitooligosaccharides whereas the monosaccharide N-acetylglucosamine is not affected (John et al., 1993). Our data indicate that chitooligosaccharide-containing signal molecules, which are probably able to control plant developmental processes are likely to be present in tobacco plants. These oligosaccharide signals are likely modified by the action of NodB. Using a plant lectin which binds specifically to chitooligosaccharides, it was recently shown that N-acetylgalcosamine-containing oligomers are present in plant secondary cell walls (Benhamou and Asselin, 1989). Since chitin is not considered to be a component of plant cell walls, these N-acetylgalcosamine-containing oligomers might be putative substrates for plant endochitinases. Indeed, an endochitinase secreted by carrot cells has been shown to play an important role in the early somatic embryo development of plants (De Jong et al., 1992). Further data suggest that lipo-chitooligosaccharides, similar to those produced by rhizobia, are present in uninfected Lathyrus plants (Spank et al., 1993). In addition, recent studies on the chemoperception of bacterial signals by plant cells have shown that these cells respond to chitooligosaccharides with four or more N-acetylgalcosamine residues by changes in the proton fluxes and in phosphorylation of specific proteins (Felix et al., 1993). The morphological abnormalities caused by the expression of the deacetylase gene nodB in transgenic tobacco support the idea that chitooligosaccharide signals have an important function in regulating plant morphogenesis.

In comparison with nodB transgenic plants, tobacco plants expressing nodA show less-pronounced but characteristic phenotypical alterations (Figure 4). In order to explain the effect on plant organogenesis caused by the nodA gene, the still unknown biochemical function of the NodA protein has to be elucidated by in vitro assays.

The nucleotide sequence of the Rhizobium nodA and nodB genes revealed an overlap of translational regulatory signals (Török et al., 1984), suggesting that the proteins encoded by these two genes are functionally linked. This gene overlap coordinates the expression of both genes very closely resulting in a ca. 30-fold higher protein level of NodA protein as compared with NodB in the bacterial cells (Schmidt et al., 1988). To ensure that both proteins are produced in the transgenic plants in roughly proper proportions, expression of nodB was driven by the 1' end of the dual TR promoter whereas nodA was expressed from the stronger 2' end (for Northern data, see Figure 2b). The putative growth factor produced by this expression of nodA and nodB in transgenic plants clearly affects the differentiation of cells leading to the formation of single bifurcated leaves and stems (Figure 3). This remarkable feature of the nodAB transgenic tobacco plants was only observed when both genes were under the control of the TR promoter. High level expression of both nodA and nodB from the CaMV 35S promoter, resulted in a different phenotype (Figure 5), which indicates that the effects on cell differentiation described above could differ according to the relative concentrations of the factors involved.

According to the biological data presently available we assume that in transgenic tobacco the NodA and NodB proteins may participate in generating N-acetylgalcosamine-containing signal molecules that regulate plant growth and development. On the other hand, one might also consider the possibility that the NodB enzyme may deacetylate the lipid-linked oligosaccharide GlcNAc-GlcNAc-pyrophosphoryl-dolichol, which is an intermediate in glycoprotein biosynthesis (Elbein and Kaushal, 1990). Such a putative inhibition of protein glycosylation could seriously affect glycoprotein function. Subsequent studies will therefore be necessary to isolate and characterize the oligosaccharide target molecules in both wild-type and transgenic plants.
Experimental procedures

Bacterial strains and plasmids

The Escherichia coli strains used were JM105 (Yanisch-Perron et al., 1985) and S17 (Simon et al., 1983). Agrobacterium tumefaciens GV3101 pMP90RK (Koncz and Schell, 1986) is a deletion derivative of strain C58 lacking the T-DNA region. Plant expression vectors pPCV701 (Koncz et al., 1987) and pPCV702 (Koncz et al., 1989) have been described.

Construction of plasmids

Recombinant DNA techniques were carried out essentially as described (Sambrook et al., 1989). Plasmid pJS201 (Schmidt et al., 1984) was used as a source of the nodA and nodB genes of Rhizobium meliloti. In pJS201 the nodA gene is contained within a 0.67 kb MboI fragment which was subcloned into the BamHI site of pUC18. The nodB gene was isolated as a 0.7 kb Real fragment and BamHI linkers or SalI linkers, respectively, were attached prior to subcloning in pUC18.

To construct pPCV702nodA and pPCV702nodB the corresponding nod coding regions were inserted into the BamHI site of the CaMV 35S plant expression vector pPCV702 (Figure 1).

For the simultaneous expression of nodA and nodB the genes were inserted into the unique SalI and BamHI sites of pPCV701, respectively. In the resulting plasmid pPCV701nodAB nodB was under mas 1' and nodA under mas 2' promoter control (Figure 1). These constructions were transformed into E. coli strain S17 and mobilized into A. tumefaciens strain GV3101 (pMP90RK) as described (Koncz and Schell, 1986).

Plant transformation and tissue culture

A. tumefaciens GV3101 strains containing the different constructions were used to infect leaf discs of Nicotiana tabacum cv. Petit Havana SR1 (Mailiga et al., 1973) as described by Horsch et al. (1985). The infected discs were then transferred to petri plates containing solid LS medium (Linsmaier and Skoog, 1965) supplemented with naphthyl acetic acid (NAA; 0.1 μg ml⁻¹), benzylaminopurine (BAP; 0.5 μg ml⁻¹), ceforan (500 μg ml⁻¹), and kanamycin (100 μg ml⁻¹). Shoots were excised and rooted on hormone-free medium in the presence of kanamycin (100 μg ml⁻¹) and ceforan (500 μg ml⁻¹). Transformed regenerated plants were self-pollinated. To follow the segregation of the T-DNA-encoded kanamycin-resistance marker gene 100 seeds were germinated on LS medium and the kanamycin-resistant progenies were counted.

DNA and RNA analysis

Genomic DNA was isolated from plant tissue as described (Dellaporta et al., 1983). The integration of nod genes into the plant genome was checked by Southern hybridization as described in Sambrook et al. (1989). Total RNA was extracted from transgenic tissues as described (Chirgwin et al., 1979). Poly(A)^+ RNA was purified by chromatography on oligo (dT) cellulose using spun columns (Pharmacia). For Northern hybridizations, samples of polyadenylated RNA were separated by electrophoresis through a 1.5% MOPS-formaldehyde gel and transferred to Hybond-N membranes (Amersham) also by procedures described by Sambrook et al. (1989). As specific probes a 0.34 kb Apal-Nhel nodA fragment and a 0.25 kb DraII-NarI nodB fragment were labeled using the random prime labeling kit from Boehringer.

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References


