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

Jürgen Schmidt[1,*], Horst Röhrig[1], Michael John[1], Ursula Wieneke[1], Gary Stacey[2], Csaba Koncz[1] and Jeff Schell[1]

[1]*Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, 50829 Köln 30, Germany, and* [2]*Center for Legume Research, Department of Microbiology and Graduate Program of Ecology, University of Tennessee, Knoxville, TN 37996-0845, USA*

Summary

The highly conserved *Rhizobium* nodulation genes *nodABC* are required to produce lipid-linked chitooligosaccharide signal molecules which elicit nodule organogenesis in roots of leguminous plants. Recently, it has been shown that NodB deacetylates chitooligosaccharides at the non-reducing terminus, so that the free amino group of the chitooligosaccharide backbone can then be acylated by a specific fatty acid. The *Rhizobium* NodA protein together with the *nodB* encoded chitooligosaccharide 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 regulation of plant development, we introduced and expressed the *Rhizobium meliloti 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 proteins to produce signal molecules modulating plant growth and organ development.

Introduction

Rhizobia elicit nodule organogenesis in roots of leguminous plants by secreting host-specific lipo-oligosaccharide signal molecules (Truchet *et al.*, 1991). These extracellular bacterial signals consist of an oligosaccharide backbone of four or five β 1,4-linked *N*-acetylglucosamine residues which carries at the non-reducing end a long-chain unsaturated fatty acyl group (Lerouge *et al.*, 1990; Schultze *et al.*, 1992; Spaink *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 chitooligosaccharide signal molecules (Spaink *et al.*, 1991). Recently, we provided evidence, that the NodB gene product is an oligosaccharide-modifying enzyme which deacetylates the non-reducing *N*-acetylglucosamine residue of chitooligosaccharides (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 meliloti nodA* and *nodB* genes separately or in combination 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 T_R promoter of mannopine synthase is outlined in Figure 1 and in Experimental procedures. To express the *R. meliloti nodA* and *nodB* genes separately or in combination, 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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* For correspondence (fax +49 221 5062 213).

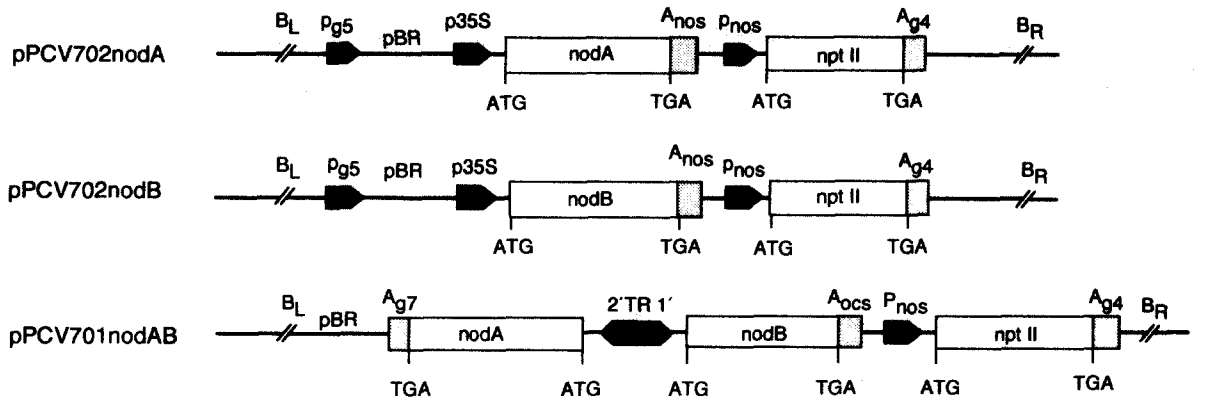

Figure 1. Schematic map of vectors used for the expression of *nodA* and *nodB* in plants.
In pPCV702nodA and pPCV702nodB the *nod* genes are under the control of the CaMV 35S promoter; pPCV701nodAB carries the *nodA* and *nodB* genes under transcriptional control of the 1',2' dual T_R promoter.
Abbreviations: Pg5, T-DNA gene 5 promoter; p35S, cauliflower mosaic virus 35S promoter; p*nos*, promoter of nopaline synthase gene; 1',2' dual T_R promoter, a plant promoter derived from mannopine synthase gene; A*nos*, A*ocs*, A_{g4} and A_{g7} are polyadenylation signals of nopaline synthase, octopine synthase, T-DNA genes 4 and 7 , respectively; B_L and B_R, left- and right-border 25 bp repeats of T-DNA; pBR, pBR322 replicon within the T-DNA; *nptII*, neomycin phosphotransferase gene of Tn*5*. Initiation and termination codons are indicated.

from which four independent transgenic lines were selected for further studies. Southern blot analyses of DNA extracted from leaves of the R_0 plants and of their F_1 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 F_1 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 pPCV702nodA 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 F_1 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 T_R promoter shows a strong hybridization signal for *nodA* (lane 3) but no detectable *nodB*-specific transcripts (lane7). 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 T_R 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).

Simultanous expression of nodA *and* nodB *driven by the dual T_R 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

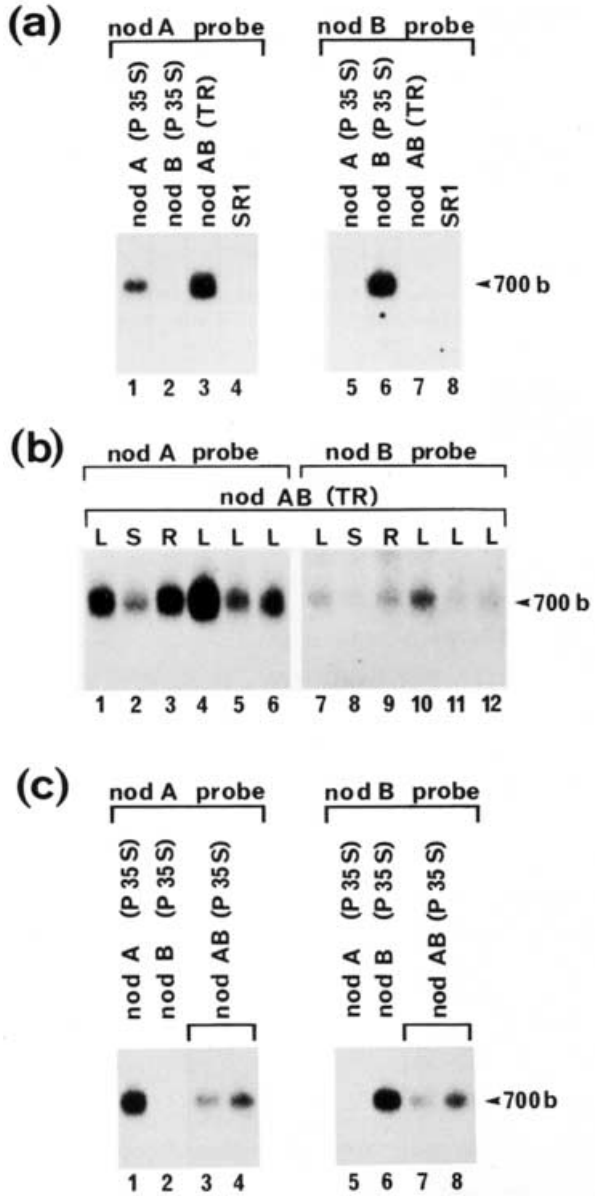

Figure 2. Identification of *nodA* and *nodB* mRNAs in transformed tobacco plants.

Transcription of the *nod* genes was under the control of the CaMV 35S promoter (p35S) or the 1',2' dual T_R 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.

expressing *nodAB* from the T_R 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 F_1 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 T_R 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 axle (Figure 3d). These phenotypic alterations described above were found in 50% of the pPCV701nodAB 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 T_R promoter, no bifurcated leaves and stems were observed with transgenic plants expressing either *nodA* or *nodB* genes alone.

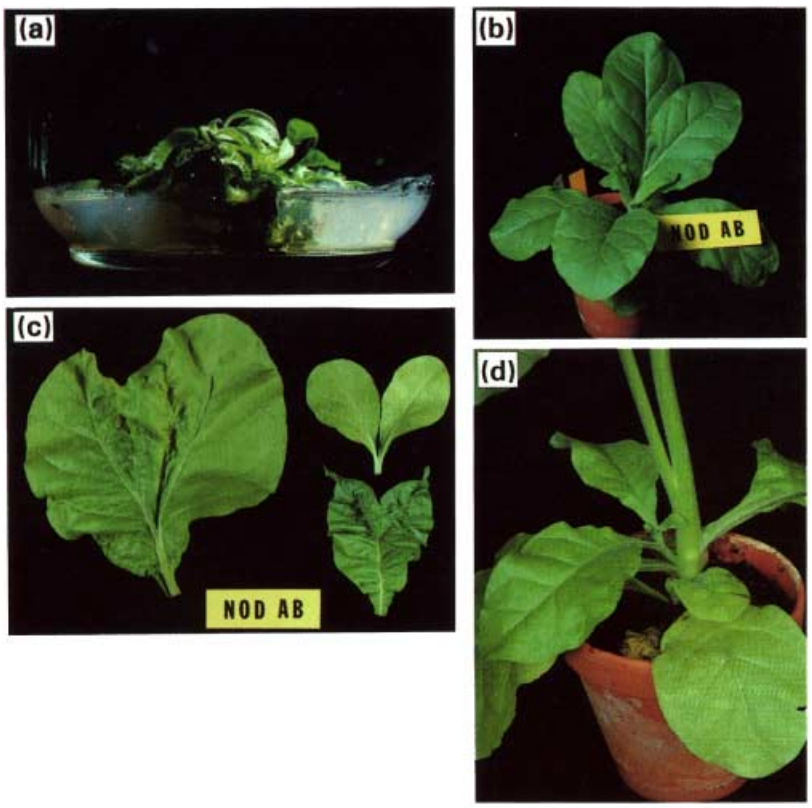

Figure 3. Transgenic tobacco plants expressing *nodA* and *nodB* genes under control of the dual T_R promoter.
(a) Development of shoots on hormone-free LS medium.
(b) Growth of the F_1 progeny of transformant pPCV701nodAB. Note the formation of a bifurcated leave.
(c) Comparison of different bifurcated leaves from independent lines of pPCV701nodAB transgenic plants.
(d) Formation of two stems emerging independently of the leaf axle.

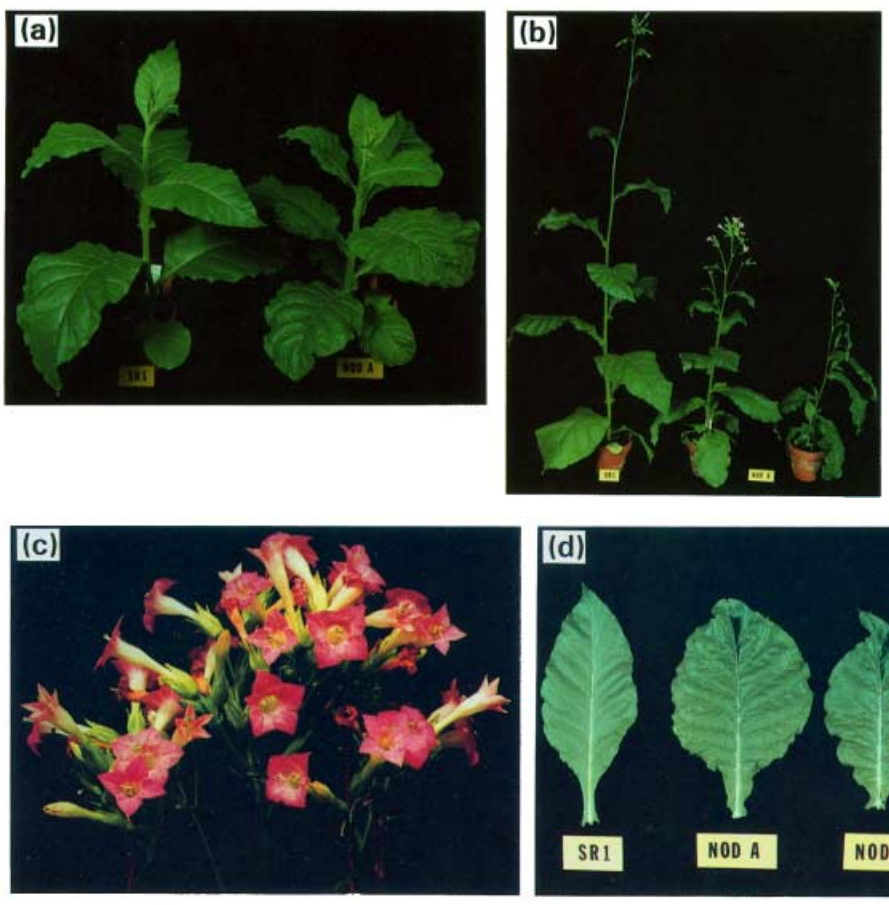

Figure 4. Separate expression of *nodA* and *nodB* genes in transgenic tobacco.
(a) Comparison of a pPCV702nodA transgenic plant (right) with a wild-type SR1 plant (left).
(b) Comparison of two independent lines of pPCV702nodB transgenic plants (right) with a wild-type SR1 plant (left).
(c) Compact inflorescence 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.

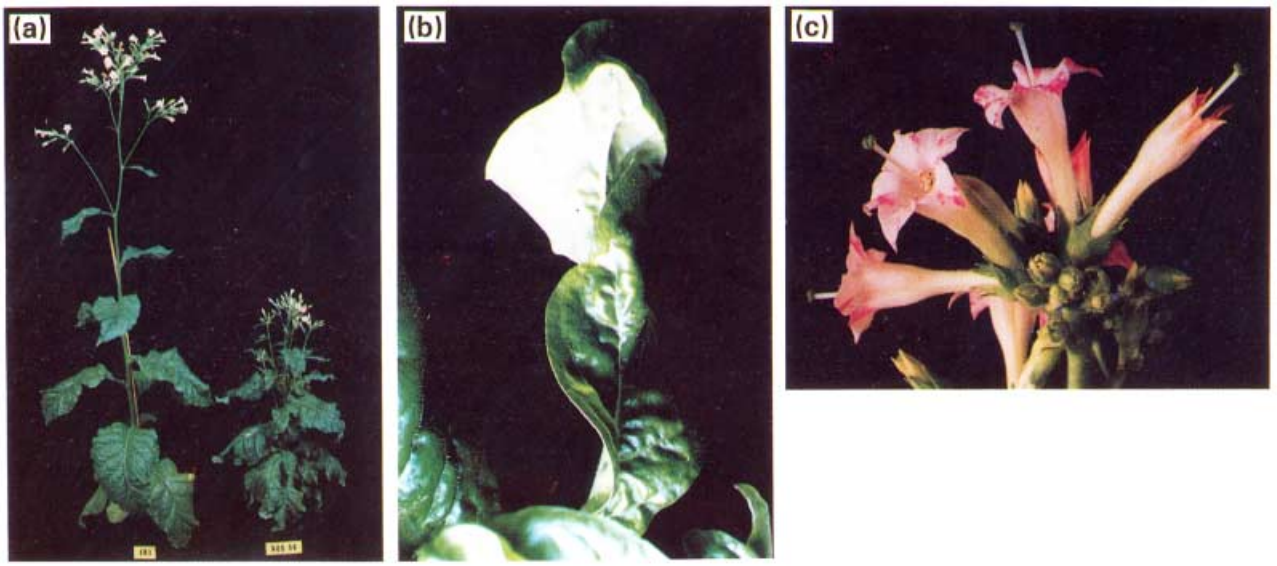

Figure 5. Transgenic tobacco plants expressing *nodA* and *nodB* genes under control of the CaMV 35S promoter. Plants were obtained by crossing of pPCV702nodA with pPCV702nodB transgenic plants.
(a) Comparison of a SR1 control plant (left) with a *nodAB* transgenic tobacco plant (right).
(b) Altered leaf morphology of an adult *nodAB* transgenic plant. Note the curling of the leaf blade.
(c) Flowers of a *nodAB* transgenic plant showing heterostyly.

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 T_R 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 F_1 transgenic plants described in this paper no obvious alteration of root morphology was observed. In comparison with the wild-type plants there

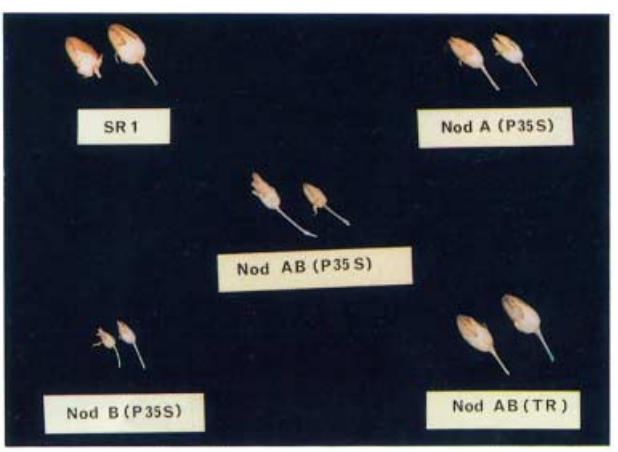

Figure 6. Comparison of seed capsules from wild-type SR1 and transgenic plants. The *nod* genes and the different plant promoters used for their expression are indicated. The seed capsules were obtained by pollination of the transgenic plants with wild-type SR1 pollen.

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 lipo-chitooligosaccharide signals, which are released by the bacteria and act on leguminous plants as nodule-inducing morphogens (Spaink *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*-acetylglucosamine 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*-acetylglucosamine-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*-acetylglucosamine-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 (Spaink *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*-acetylglucosamine 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 chitoooligosaccharide 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 insure 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 T_R 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 T_R 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 differentiaton 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*-acetylglucosamine-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 *Mbo*II fragment which was subcloned into the *Bam*HI site of pUC18. The *nodB* gene was isolated as a 0.7 kb *Rsa*I fragment and *Bam*HI linkers or *Sal*I linkers, respectively, were attached prior to subcloning in pUC18.

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

For the simultanous expression of *nodA* and *nodB* the genes were inserted into the unique *Sal*I and *Bam*HI 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 (Maliga *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}), claforan (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 claforan (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 *Apa*I–*Nhe*I *nod*A fragment and a 0.25 kb *Dra*III–*Nar*I *nod*B fragment were labeled using the random prime labeling kit from Boehringer.

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