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Expression of Soybean Nodulin 26 in Transgenic Tobacco. Targeting to the Vacuolar Membrane and Effects on Floral and Seed Development

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Nodulin 26 is an integral membrane protein of the symbiosome membrane of nitrogen-fixing soybean nodules. We expressed a nodulin 26 cDNA in transgenic tobacco (TN26 tobacco) under the control of the cauliflower mosaic virus 35S promoter to study subcellular targeting and the physiological effect(s) of its expression. Based on Northern and Western blots, the expression of nodulin 26 mRNA and protein in transgenic plants is high in apical shoot sections, flowers, and stems, low in mature leaves, and absent in roots. Western blot analysis revealed high levels of transgenic nodulin 26 protein in tonoplast membranes. In contrast, nodulin 26 protein was not found in isolated plasma membranes, the soluble fraction, nor in chloroplast and mitochondria-enriched membrane fractions. About 50–60% of the flowers and pods from TN26 tobacco plants abscised prematurely. Seed capsule size and seed fill per capsule from the remainder of surviving flowers were about 50% of that of control plants. Pollen viability was found to be normal, but flowers from TN26 tobacco plants showed shorter anther filaments compared with control plants. Normal seed production and capsule size was restored by manually crossing the stigmas from TN26 plants with isolated pollen from either transgenic or control plants. Thus, the aberrant filament growth could have resulted in the reproductive defects associated with the plants.

INTRODUCTION

Infection of soybean roots by *Bradyrhizobium japonicum* bacteria leads to the formation of a nitrogen fixation organ known as the nodule. The bacteria are endosymbiotic and become enclosed in a specialized organelle in the plant cytosol known as the "symbiosome" (Roth *et al.*, 1988). The symbiosome membrane delimits this organelle and serves to: 1) control the flux of metabolites and nutrients between the plant host and the symbiont (Day and Udvardi, 1993) and 2) to protect the bacteria from defense responses of the plant host (Werner *et al.*, 1985). During the formation of the nodule, a number of nodule-specific proteins (termed "nodulins," reviewed by Franssen *et al.*, 1992) are expressed. Some of these are specifically targeted to the symbiosome membrane (Fortin *et al.*, 1985). Among these is an integral membrane protein, nodulin 26, that is a major protein component of the symbiosome membrane (Fortin *et al.*, 1987; Weaver *et al.*, 1991; Miao *et al.*, 1992).

Based on its deduced amino acid sequence, nodulin 26 has been demonstrated to be a member of the major intrinsic protein (MIP)[1] family (Sandal and Marcker, 1988; Shiels *et al.*, 1988) [reviewed in Reizer *et al.* (1993)]. Members of this family share 30–40% se-

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[1]Abbreviations used: BSA, bovine serum albumin; BTP, bis-tris propane; CaMV 35S, cauliflower mosaic virus 35S promoter; DTT, dithiothreitol; E_7 antibodies, nodulin 26-specific antibodies prepared against isolated nodulin 26 protein; HEPES, *N*-(2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MIP, major intrinsic protein; MS medium, Murashige and Skoog medium; NC, negative control transgenic tobacco plants transformed with the pGDW31 binary vector without nodulin 26 cDNA insert; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; pGNod26, pGDW31 containing the nodulin 26 cDNA; PMSF, phenylmethylsulfonyl fluoride; T-DNA, DNA sequences in pGDW31 that are transferred to the plant during *Agrobacterium* transformation; TIP, tonoplast intrinsic protein; TN26, transgenic tobacco plants transformed with the pGNod26; W38, control untransformed *N. tabacum* cv. Wisconsin 38 plants.

quence identity and are proposed to be integral membrane proteins with six membrane-spanning domains with hydrophilic NH_2- and COOH-terminal domains facing the cytoplasm [reviewed in Reizer *et al.* (1993)]. At least 18 members of this family have been documented (Reizer *et al.*, 1993). Functional activities have been described for some of these proteins and include various transport activities such as ion channel (Ehring *et al.*, 1990), water channel (van Hoek and Verkman, 1992; Preston *et al.*, 1992; Maurel *et al.*, 1993) and glycerol transport activities (Heller *et al.*, 1980; Maurel *et al.*, 1994). Recently nodulin 26 was shown to form ion channels upon reconstitution into planar lipid bilayers (Weaver *et al.*, 1994), but its physiological function in the symbiosome membrane is not yet known.

In the present study we have constructed transgenic tobacco plants that express nodulin 26 under the control of the cauliflower mosaic virus 35S promoter and have investigated its subcellular localization and the physiological effects of expression of this protein on the growth and development of tobacco plants.

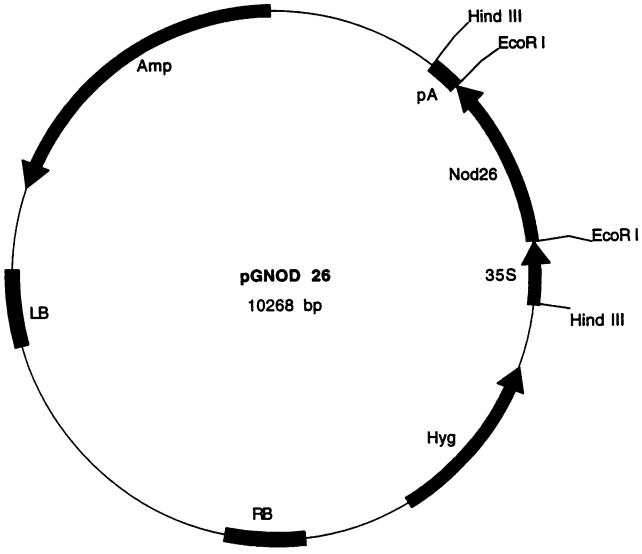

Figure 1. Construction of a binary vector harboring nodulin 26 cDNA insert (pGNod26) for plant transformation. Nod 26, full length soybean nodulin 26 cDNA; pA, poly A terminal sequence; 35S, cauliflower mosaic virus 35S promoter; LB and RB, left and right border of *A. tumefaciens* T-DNA; Amp and Hyg, ampicillin and hygromycin resistance genes.

MATERIALS AND METHODS

Molecular Cloning Techniques

To construct vectors for nodulin 26 expression, it was first necessary to obtain a full length cDNA encoding the protein. A λgt 11 cDNA library constructed from mRNA of 14-day-old soybean root nodules (a gift of Dr. C. Sengupta-Gopalan of New Mexico State University, Las Cruces) was screened for a nodulin 26 cDNA clone by site-directed antibodies against nodulin 26 (Weaver *et al.*, 1991), as well as with nodulin 26-specific oligonucleotide probes (5′-GAGGAAAAAGGTCATTAT-3′ and 5′-GGAGGCAGCACGGCCTTT-3′). A partial, 524-bp cDNA clone (J44) encoding nodulin 26 (corresponding to the sequence between the 3′-end *Eco*RI site and an internal *Eco*RI site within the coding region of nodulin 26 cDNA) was obtained. Another cDNA clone encoding the remaining nodulin 26 sequences between 5′-end *Eco*RI site and the internal *Eco*RI site (Sandal and Marcker, 1988) was kindly provided by Dr. N. Sandal and Dr. K. Marcker of the University of Aarhus, Denmark. A full length nodulin 26 cDNA clone was constructed by ligation of the two clones, and was confirmed by dideoxynucleotide sequence analysis.

Generation of Transgenic Plants

The full-length nodulin 26 cDNA was removed by partial digestion with *Eco*RI. This fragment was used to generate the binary plant transformation plasmid pGNod 26 (see Figure 1) from the pGDW31 plasmid by the general approach previously described (Roberts *et al.*, 1992). The nodulin 26 cDNA is expressed under the control of the cauliflower mosaic virus 35S promoter in this construct. Production of transgenic plants was done by *Agrobacterium tumefaciens* transformation of tobacco (*Nicotiana tabacum* cv. Wisconsin 38) by the general method of Horsch *et al.* (1985) as previously described (Roberts *et al.*, 1992). Hygromycin resistance was used as a selectable marker. F_0 plants generated from tobacco callus, including negative control (NC) transgenic plants transformed with the pGDW31 binary vector alone (i.e., no nodulin 26 cDNA) and transgenic plants (TN26) transformed with pGNod 26, were transferred and grown in a greenhouse. F_1 seed were collected and stored at 4°C. To test for Mendelian segregation, F_1 progenies of transgenic tobacco plants were germinated on MS media with 20 μg/ml hygromycin B (Calbiochem, La Jolla, CA), and the ratio of hygromycin resistant to sensitive seedlings was recorded.

The basic medium for the germination of tobacco pollen was the same as described in Spena and Schell (1987) except for the addition of 20 μg/ml hygromycin B. Pollen from single flowers of different tobacco lines was collected and germinated in 1 ml of medium for 2–2.5 h at room temperature. Pollen viability was checked by staining with KI (Clark, 1981). For crossing, single unopened flowers (12–13-wk-old plants) were chosen, anthers were removed, and the desired pollen dusted onto the stigma of the emasculated flowers.

Analysis of Nodulin 26 Expression in Transgenic Plants

Stable transformation of transgenic tobacco with nodulin 26 was confirmed by PCR analysis of genomic DNA samples. Genomic DNA was isolated from young, expanding leaves of 14-wk-old tobacco plants by the cetyltrimethylammonium bromide method (Rogers and Bendich, 1988). Genomic DNA samples (50 μg) were digested with 200 units of *Hin*dIII and 100 μg of DNase-free RNase A at 37°C overnight. PCR was carried out with specific oligonucleotide primers. The forward primer (5′-GGGATCCATGGCTGATTATTCAGCAGG-3′) contained the first 18 nucleotides of the coding sequence of nodulin 26 cDNA with additional *Bam*HI and *Nco*I sites fused to the 5′-end. The reverse primer (5′-GAGGATCCCCGGGTACCGAG-3′) contained the sequence between the *Sac*I and *Xba*I sites within the polylinker region of pRT101 (Topfer *et al.*, 1987). One μg of each primer was used in all PCR reactions with *Hin*dIII-digested genomic tobacco DNA (1 μg). Fifty cycles of PCR were performed with 4 units of *Taq* DNA polymerase in 50 mM Tris-HCl, pH 9.0, 50 mM KCl, 1% (vol/vol) Triton X-100, 2.5 mM $MgCl_2$, and 0.2 mM of each nucleotide triphosphate. Each cycle consisted of 94°C for 30 s for denaturation, 72°C for 30 s for annealing, and 45°C for 1 min for extension.

Northern blot analysis was done by the general method of Sambrook *et al.* (1989) on total RNA samples isolated from different tissues by the method of Chirgwin *et al.* (1979). RNA samples were resolved by electrophoresis on a 1% (wt/vol) agarose gel in 20 mM sodium phosphate, pH 7.2, 6% (vol/vol) formaldehyde. RNA was transferred to a Gene-Screen Plus nylon membrane (DuPont, Wilmington, DE), and was incubated at 42°C for 1–3 h with 10 ml of 50% (vol/vol) formamide, 5× Denhardt's reagent (Sambrook *et al.*, 1989), 6× SSC (1× SSC, standard sodium citrate buffer, 0.15 M NaCl and 15 mM sodium citrate), 2% (wt/vol) SDS and 100 μl of 5 μg/μl salmon sperm DNA (hybridization buffer). The J44 partial nodulin 26 cDNA clone (see above) was labeled with [α-^{32}P]dCTP by using a nick-translation kit and the manufacturer's protocol (Promega, Madison, WI). This probe (10^7 cpm) was hybridized with the RNA blot membrane in 10 ml of fresh hybridization buffer for 16 h at 42°C. The blot was washed at 37°C: once with 2× SSC, once with 1× SSC containing 0.5% (wt/vol) SDS, and once with 0.1× SSC containing 0.5% (wt/vol) SDS. Autoradiography was done at −80°C with an intensifying screen.

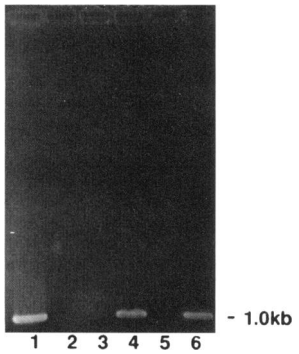

Figure 2. PCR amplification of nodulin 26 gene from transgenic plant genomic DNA. All DNA samples were digested with *Hin*dIII before PCR amplification with nodulin 26 specific primers as discussed in MATERIALS AND METHODS. Lane 1, positive control, the pGNod26 plasmid (Figure 1); lane 2, genomic DNA from untransformed W38 tobacco leaves; lane 3, genomic DNA from NC-1 transgenic tobacco leaves (F_0); lane 4, genomic DNA from TN26-1 transgenic tobacco leaves (F_0); lane 5, genomic DNA from NC-1 transgenic tobacco leaves (F_1); lane 6, genomic DNA from TN26-1 transgenic tobacco leaves (F_1). The position of the 1 kb DNA marker is shown.

Immunochemical Methods

Nodulin 26 antibodies against purified nodulin 26 were prepared by the general approach described by Weaver *et al.* (1991). Nodulin 26 was isolated from symbiosome membranes by SDS-PAGE in a continuous gel electrophoresis cell (Bio-Rad model 491 prep. cell). Polyclonal rabbit antibodies (E_7) were prepared against isolated nodulin 26 by the immunization schedule described by Weaver *et al.* (1991). Reactivity with nodulin 26 was verified by Western blot analysis (Weaver *et al.*, 1991). The antibody showed specificity for nodulin 26 and showed no cross reactivity with other proteins in extracts of soybean or tobacco tissues. Preimmune sera or IgG was used as a negative control in all Western blot experiments.

Subcellular Fractionation

All procedures for extraction and fractionation were carried out at 4°C. Fresh tissue (5–10 g) from the apical 2 cm of the shoots of 12–13-wk-old tobacco were ground in two volumes of 50 mM HEPES-BTP, pH 7.4, 250 mM sorbitol, 6 mM EGTA, 1 mM DTT, 0.1% (wt/vol) BSA, 1 mM PMSF, and 2 μg/ml leupeptin. The homogenate was filtered through one layer of Miracloth (crude extract), and was centrifuged at 500 × *g* at 4°C for 10 min. The 500 × *g* supernatant was then centrifuged at 13,000 × *g* at 4°C for 10 min. The pellet, which is enriched in chloroplast and mitochondria, was saved. The 13,000 × *g* supernatant was centrifuged at 100,000 × *g* at 4°C for 1 h to separate the soluble (supernatant) and microsomal fractions (100,000 × *g* pellet).

Tonoplast membranes were purified from the microsomal membrane fraction according to Ward *et al.* (1992). Briefly, the 100,000 × *g* pellet was resuspended in 0.5 ml of 25 mM HEPES-BTP, pH 7.2, 250 mM sorbitol, 1 mM DTT 0.1 mM PMSF, and 2 μg/ml leupeptin (resuspension buffer). The sample was loaded onto 5 ml of 6% (wt/vol) dextran and was centrifuged at 70,000 × *g* at 4°C for 2.5 h. The interface between the aqueous and dextran phases was removed and diluted with 4 volumes of resuspension buffer. The sample was centrifuged at 85,000 × *g* at 4° for 30 min. The final pellet, which contains purified tonoplast membranes, was resuspended in 150 μl of resuspension buffer. Plasma membranes were purified directly from tobacco samples by the two-phase partitioning protocol of Larsson *et al.* (1987). The purity of the tonoplast and plasma membrane samples were verified by marker enzyme assays (Graham, 1993): catalase for peroxisomes; NADPH-cytochrome *c* reductase for endoplasmic reticulum; succinate dehydrogenase for mitochondria; and 5′-nucleotidase for the plasma membrane. Chlorophyll was determined according to Arnon (1947). Acid phosphatase was chosen as the marker enzyme of the tonoplast and was assayed as reported in Höfte and Chrispeels (1992). Identical amounts of the various membrane fractions were tested for nodulin 26 by Western blot analysis with E_7 antibodies. Protein analysis was done by the method of Bradford (1976).

RESULTS

Construction of Transgenic Tobacco Plants that Express Nodulin 26

Seven separate lines of negative control (NC) transgenic tobacco plants were obtained from *Agrobacterium*-mediated transformation with the pGDW31 plasmid lacking a nodulin 26 cDNA insert. Nine separate nodulin 26 transgenic tobacco lines (TN26) were generated from independent transformation with pGNod26 (Figure 1). To test for genomic intergration of the nodulin 26 construct, *Hin*dIII-digested genomic DNA from untransformed and transgenic tobacco plants was analyzed by PCR with oligonucleotide primers specific for the nodulin 26 nucleotide sequence. Only one PCR product, of the expected length of 986 bp, was observed from reactions of genomic DNA samples of both F_0 and F_1 generations of all TN26 tobacco plants. This product was not observed in reactions of genomic DNA of untransformed W38 tobacco or NC tobacco plants (Figure 2). The results indicate that the nodulin 26 cDNA was stably integrated into the genome of TN26 transgenic plants.

To address the expression of nodulin 26 mRNA in transgenic tobacco, total RNA isolated from young leaves was subjected to Northern blot analysis with a nodulin 26 cDNA probe (Figure 3A). Total RNA from soybean nodules was used as a positive control and revealed a single band of 960 nucleotides (lane 1), corresponding to the mRNA of native nodulin 26. A similar transcript was detected by the probe in TN26 transgenic lines (Figure 3A). This mRNA was not detected in NC transgenic or untransformed (W38) tobacco plants (Figure 3A). Thus, the nodulin 26 transcripts were only detected in TN26 transgenic tobacco plants transformed with nodulin 26 cDNA and did not hybridize with any endogenous mRNAs.

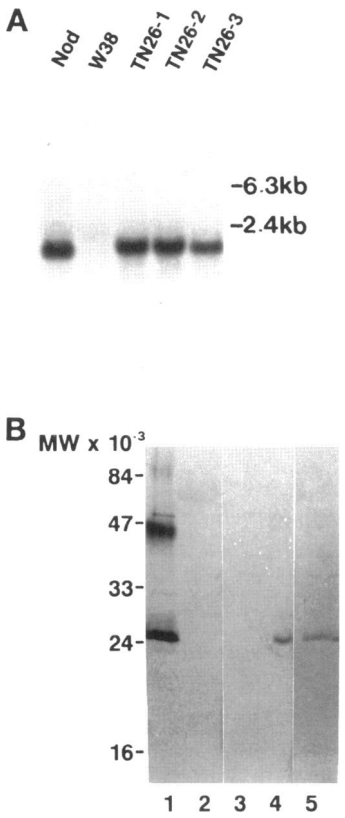

Figure 3. Expression of nodulin 26 in transgenic tobacco. (A) Northern blot with nodulin 26-specific cDNA probe. Each lane was loaded with 20 μg of total RNA from: soybean nodules (Nod); leaves of untransformed tobacco (W38); the leaves of three separate TN26 transgenic tobacco lines (TN-1, -2, and -3). (B) Western blot analysis. Samples (20 μg protein/lane) were separated by SDS-PAGE on a 15% (wt/vol) polyacrylamide gel, were transferred to nitrocellulose, and were analyzed with E_7 nodulin 26-specific antibodies. Apices are defined as the apical 2 cm of the tobacco plant. Lane 1, symbiosome membranes from soybean nodules (the upper band represents dimerized nodulin 26); lane 2, crude extract of apices from NC-1 tobacco plants; lane 3, microsomal membranes (100,000 $\times$ g pellet) from apices of NC-1 tobacco plants; lane 4, crude extract of apices from TN26-1 tobacco plants; lane 5, microsomal membranes (100,000 $\times$ g pellet) from apices of TN26-1 tobacco plants. All data were obtained with tissues from 12-wk-old tobacco plants.

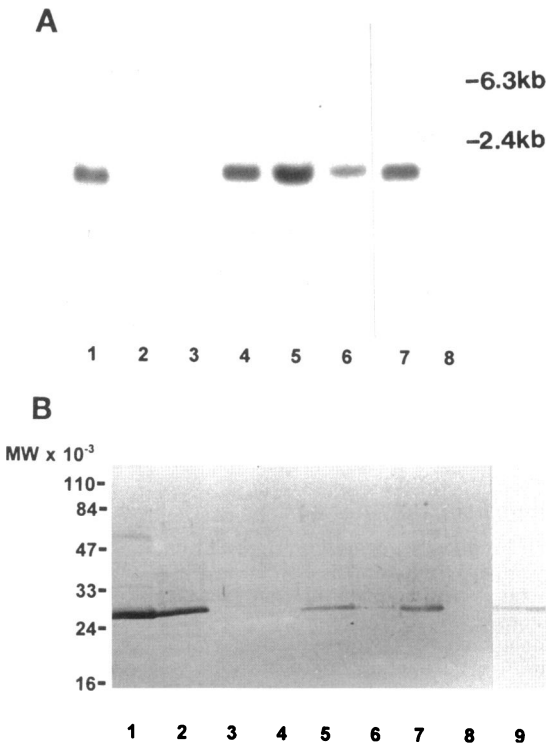

Figure 4. Tissue distribution of nodulin 26 mRNA and protein expressed in 12-wk-old transgenic tobacco plants. (A) Northern blot analysis. Total RNA (20 μg/lane) from various tissues were analyzed as described in the MATERIALS AND METHODS. Lane 1, soybean nodules; lane 2, apices from W38 tobacco; lane 3, apices from NC-1 transgenic tobacco; lanes 4, TN26-1 tobacco flowers; lane 5, TN26-1 tobacco apices; lane 6, TN26-1 tobacco leaves; lane 7, TN26-1 tobacco stems; lane 8, TN26-1 tobacco roots. (B) Western blot analysis. Extracts (20 μg protein/lane) from the various tissues were analyzed for nodulin 26 with E_7 antibodies as described in MATERIALS AND METHODS. Lane 1, symbiosome membrane from soybean nodules; lane 2, soybean nodule extract; lane 3, apices from W38 tobacco plants; lane 4, apices from NC-1 transgenic tobacco plants; lane 5, TN26-1 tobacco flowers; lane 6, TN26-1 tobacco leaves; lane 7, TN26-1 tobacco apices; lane 8, TN26-1 tobacco roots; lane 9, TN26-1 tobacco stems.

To determine whether nodulin 26 protein is expressed in transgenic plants, extracts and 100,000 $\times$ g membrane fractions obtained by differential centrifugation were analyzed by Western blot with a nodulin 26-specific antibody (E_7). No signal was observed in crude or microsomal fractions of NC plants (Figure 3B, lanes 2 and 3). However, the nodulin 26 antibodies detected a 27,000 M_r band in membrane fractions of TN26 plants (Figure 3B, lanes 4 and 5) that co-migrates with native nodulin 26 from soybean symbiosome membranes (Figure 3B, lane 1). The minor band of about 47,000 represents the dimerization of nodulin 26 which commonly occurs (Weaver *et al.*, 1991). No reactive bands were observed when antibodies were replaced with preimmune sera or IgG. These results show that nodulin 26 protein is stably expressed in transgenic tobacco.

Tissue and Subcellular Distribution of Nodulin 26

By Northern blot analysis, nodulin 26 mRNA was expressed most strongly in apices, flowers, and stems. Levels were lower in leaves of TN26 tobacco plant, and there was no apparent nodulin 26 in the RNA sample from roots (Figure 4) even though the CaMV 35S promoter normally is active in transgenic root tissue (Williamson *et al.*, 1989). Figure 4B shows that the expression of nodulin 26 protein parallels the expression of mRNA, with high levels of nodulin 26 protein occurring in apices, flowers, and stems, low levels in mature leaves, and no detectable protein in roots.

Nodulin 26 has been demonstrated to be a major membrane protein of the symbiosome membrane of soybean root nodules (Weaver *et al.*, 1991). To localize nodulin 26 protein expressed in transgenic tobacco plants, subcellular membrane fractionation and Western blot analyses were done. The purity of the membrane fractions was verified by marker enzyme assays (Table 1). Western blot analysis showed no significant nodulin 26 signal in soluble (100,000 $\times$ g supernatant),

Table 1. The activity of membrane marker enzyme of various sample fractions from TN26-1 transgenic tobacco apices

Sample[a]	Chlorophyll (mg/l)	Catalase (nmol/min/ μg protein)	Succinate dehydrogenase (ΔA_{490}/min/ μg protein)	5'-Nucleotidase (pmol/min/μg protein)	NADPH-cytochrome *c* reductase (ΔA_{550}/min/μg protein)	Acid phosphatase (pmol/h/μg protein)
Crude	3.60	6.40	0.27	36.2	0.57	ND[b]
Mit/chlor	2.90	0.90	0.24	ND	0	ND
MM	0.11	5.10	0.01	26.6	0.45	0.85
PM	0	0	0	25.9	0	0.01
TM	0	0	0	0	0	0.70

[a] Crude, crude extract of tobacco apices in grinding buffer; Mit/chlor, mitochondria and chloroplast fraction (13,000 × *g* pellet); MM, microsomal membrane (100,000 × *g* pellet); PM, purified plasma membranes; TM, purified tonoplast membranes (see MATERIALS AND METHODS for details on membrane preparation).

[b] ND, not determined.

plasma membrane, and mitochondria and chloroplast membrane-enriched fractions (13,000 × *g* pellet) from TN26 transgenic tobacco plants (Figure 5). In contrast, nodulin 26 was readily detected in purified tonoplast membranes from TN26 plants, but not in tonoplast membranes from untransformed or NC plants (Figure 5). This result shows that among the purified membrane fractions tested, nodulin 26 is only found in the tonoplast membranes of transgenic tobacco plants.

Phenotypical Properties of Transgenic Tobacco

To assess the physiological impact of nodulin 26 expression on transgenic tobacco, we compared the growth and development of TN26 plants with untransformed tobacco and NC plants. There was no visible difference between the vegetative growth of any of the TN26 plant lines and control plants (data not shown). However, there are marked differences between TN26 and control plants with respect to flower and seed capsule development (Table 2). About 50% of the flowers from TN26 transgenic tobacco plants abscised prematurely after opening, compared with abscission frequencies of less than 7% for W38 and NC transgenic tobacco. Further, both seed fill per capsule and seed capsule size for TN26 plants were only about 50% of that of W38 and NC tobacco plants (Figure 5A, Table 2). As shown in Table 2, all nine lines of TN26 transgenic tobacco showed similar phenotypical properties. Similarly, these phenotypes were not observed in any of the seven lines of NC control plants. Therefore, the phenotypical properties of TN26 plants seem to result from the expression of nodulin 26 rather then positional effects of T-DNA insertion.

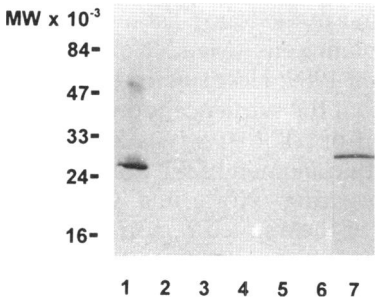

Figure 5. Western blot analyses of various membrane fractions of 12-wk-old tobacco plants. Various membrane fractions were separated by SDS-PAGE on a 15% (wt/vol) polyacrylamide gel and were analyzed by Western blot with nodulin 26-specific antibodies. Lane 1, symbiosome membranes from soybean nodules; lane 2, purified tonoplast membranes from untransformed tobacco apices; lane 3, purified tonoplast membranes from NC-1 tobacco apices; lane 4, soluble, membrane-free fraction (100,000 × *g* supernatant) from TN26-1 tobacco apices; lane 5, 13,000 × *g* pellet containing mitochondria and chloroplasts from apices of TN26-1 tobacco; lane 6, plasma membrane fraction purified by two-phase partitioning from apices of TN26-1 tobacco apices; lane 7, purified tonoplast membranes from TN26-1 tobacco apices. Five μg protein of each tonoplast membrane sample was loaded; 20 μg protein of all other TN-26 membrane samples were loaded.

The lower amount of seed per capsule may be attributed to poor fertilization in TN26 plants. However, pollen from TN26 plants was indistinguishable from control pollen with respect to in vitro germination frequency (>90%) and viability as determined by KI-staining. However, an examination of the flowers of TN26 plants showed that the anther filaments were shorter, resulting in a localization of anthers below the stigma compared with control tobacco flowers (Figure 6B). The shorter filament might result in a lower frequency of pollination, and thus lower fertilization in TN26 transgenic flowers. This possibility was tested by manual pollination experiments. Single unopened flowers were chosen from either W38, NC, or TN26 transgenic tobacco plants, the anthers were removed, and the flowers were manually pollinated. Capsules resulting from manual pollination treatments showed normal size and seed fill, even in the case of manual fertilization of TN26 stigmas with TN26 pollen (Figure

Table 2. Flower/pod abscission and seed fill of untransformed and transgenic tobacco

Plant line[a]	Premature abscission of flowers (%)[b] F_0	F_1	Seeds/capsule[c]
W 38	2.9 ± 1.8[d]	1.6 ± 0.2	1818 ± 180
NC-1	4.1 ± 0.4	2.1 ± 0.4	1820 ± 91
NC-2	4.4 ± 0.8	5.5 ± 0.4	1977 ± 100
NC-3	6.6 ± 2.5	5.4 ± 0.6	1555 ± 180
NC-4	5.4 ± 0.4	4.7 ± 0.3	2007 ± 120
NC-5	4.9 ± 1.9	6.9 ± 0.0	1966 ± 40
NC-6	3.6 ± 0.4	2.4 ± 0.0	2056 ± 100
NC-7	5.1 ± 0.4	6.1 ± 0.7	1728 ± 910
TN26-1	44.6 ± 3.0	42.9 ± 4.1	700 ± 62
TN26-2	56.6 ± 15.0	56.0 ± 7.7	780 ± 83
TN26-3	46.6 ± 2.9	39.5 ± 3.8	750 ± 71
TN26-4	48.0 ± 4.7	47.0 ± 4.3	915 ± 80
TN26-5	49.0 ± 5.8	48.0 ± 4.2	854 ± 60
TN26-6	47.0 ± 3.6	45.0 ± 6.4	901 ± 70
TN26-7	48.0 ± 6.4	48.0 ± 2.9	810 ± 30
TN26-8	48.0 ± 3.2	50.0 ± 0.5	894 ± 90
TN26-9	48.0 ± 4.9	48.0 ± 8.6	921 ± 100

[a] W 38, untransformed tobacco; NC-1, NC-2, NC-3, NC-4, NC-5, NC-6, and NC-7, transgenic tobacco lines transformed with pGDW31 vector only; TN26-1, TN26-2, TN26-3, Tn26-4, TN26-5, Tn26-6, TN26-7, TN26-8, and TN26-9, transgenic tobacco lines transformed with the pGNod26 (Figure 1).

[b] Data represent average (nine plants per analysis). Premature abscission of flowers was determined by marking flowers or flower buds on a given shoot, then counting the remainder of seed capsules on the same shoot after 3 wk.

[c] Seed numbers per capsule were the average of 10 seed pods.

[d] Standard error.

6C). Thus, the smaller seed capsule size and reduced seed number in TN26 transgenic tobacco plants appear to result from inadequate pollination, possibly due to the reduced growth of anther filaments.

DISCUSSION

In the present work, *Agrobacterium*-mediated transgenic tobacco plants were constructed that express soybean nodulin 26. The nodulin 26 cDNA was stably incorporated into the tobacco genome and was expressed differentially in tissues, ranging from high expression in the shoot apex, flowers, and stems, to no detectable expression in the root. Subcellular fractionation studies indicate that transgenic nodulin 26 is localized in the tonoplast membrane of the vacuole. Expression of nodulin 26 did not appear to affect the vegetative growth of the plant, but did affect seed production, seed pod size, and flower abscission. The lower seed production appears to result from a lower pollination frequency. This is supported by the observation of shorter anther filaments in TN26 flowers, as well as by the observation that normal seed fill and seed capsule size could be restored by manual pollination of TN26 stigmas. Thus, expression of nodulin 26 in transgenic tobacco appears to alter flower and seed development, presumably through its membrane channel activity.

The biogenesis of the symbiosome of legume root nodules is a complex event that involves the initial invagination of the invading rhizobium bacterium in the infection thread membrane. This is followed by massive membrane biosynthesis and trafficking from Golgi-derived vesicles [reviewed by Mellor and Werner (1987)]. Thus, it has been proposed that the symbiosome membrane is derived from different host membranes (Roth and Stacey, 1989) and has properties of both the vacuolar membrane (Mellor, 1989), as well as the plasma membrane (Blumwald *et al.*, 1985; Udvardi and Day, 1989; Perotto *et al.*, 1991). Several nodulin proteins, including nodulin 26, are targeted to this membrane (Fortin *et al.*, 1985, 1987; Cheon *et al.*, 1994), probably via endoplasmic reticulum and Golgi-derived vesicles (Miao *et al.*, 1992; Cheon *et al.*, 1994). The structural determinants for targeting to the symbiosome membrane are unknown.

In the model described by Mellor (1989), the symbiosome is proposed to be a nodule-specific analogue of the vacuolar compartment and is thought to be a lytic organelle. This is supported by the observation of vacuole-like hydrolytic enzymes found within the peribacteroid space [reviewed by Werner (1992)]. The observation that nodulin 26 is localized on the tonoplast of transgenic tobacco plants supports this observation and suggests that nodulin 26 may be a nodule-specific form of the TIP subfamily (Reizer *et al.*, 1993). TIPs are found in the tonoplast membrane of vacuoles of various plant cells (Maeder and Chrispeels, 1984; Johnson *et al.*, 1989; Höfte *et al.*, 1992). Previous work has shown that the sequence found within a 33-amino acid segment of αTIP (residues 209–241), which contains the sixth transmembrane domain, is sufficient for tonoplast targeting (Höfte and Chrispeels, 1992). A comparison of the aligned region of nodulin 26 shows a high degree of sequence homology, including 35% sequence identity and predominantly conservative sequence substitutions found within the sixth transmembrane domain (Reizer *et al.*, 1993). This may help account for the targeting of nodulin 26 to this location upon expression in transgenic plants. Short peptide targeting sequences for soluble vacuolar proteins have been described, however the mechanism(s) for vacuolar membrane protein targeting in plants have yet to be defined [reviewed by Chrispeels and Raikhel (1992) and Nakamura and Matsuoka (1993)].

The major phenotypical change that we have observed in TN26 transgenic tobacco plants is abnormal flower morphology (shorter anther filaments), premature abscission of flowers, and lower seed fill and

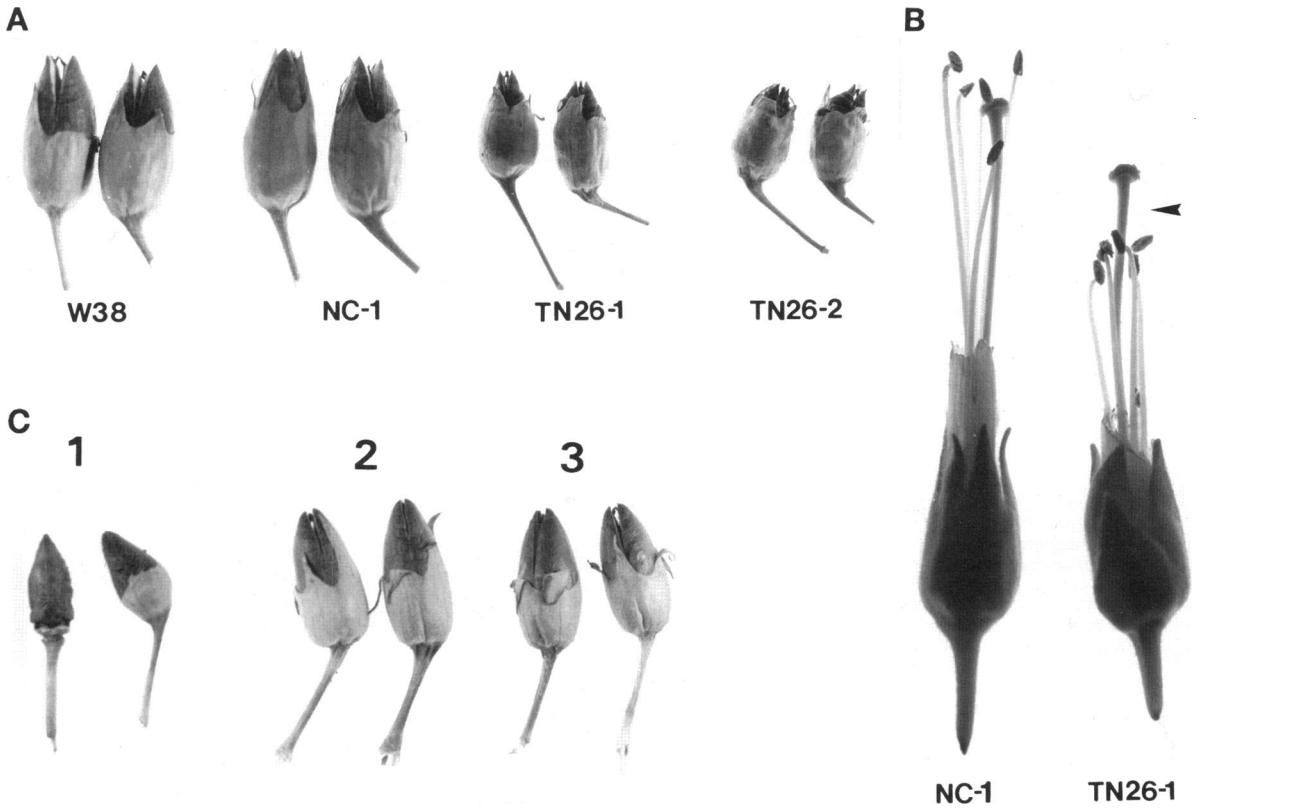

Figure 6. Comparison of seed capsules and flowers of transgenic and untransformed tobacco. (A) Representative, mature seed capsules and flowers of control (W38 and NC-1) and nodulin 26 transgenic plants (TN-1 and -2). (B) Representative control (NC-1) and nodulin 26 transgenic (TN26-1) flowers. The arrowhead indicates the shorter anther filaments below the stigma in TN26 transgenic tobacco flower. (C) Seed capsules after self-pollination and manual cross fertilization. Shown are seed pods produced by: 1, self-pollination of TN26-1 tobacco plants showing reduced capsule size; 2, self-pollination of untransformed plants showing normal capsule size and seed fill; and 3, manual pollination of TN26-1 flowers with pollen from the same flower.

smaller seed capsules. Based on manual pollination experiments, we have shown that the pollen of TN26 is viable and a decrease in delivery of pollen to the TN26 stigma is the likely reason for decreased seed fill and pod size. The higher rate of abscission of TN26 flowers may also be a result of a decrease in pollination. Failure to pollinate flowers results in premature abscission of flower pedicels (Fitting, 1911). Based on studies of mutant plants, this process has been proposed to be mediated by ethylene (Lanahan *et al.*, 1994).

How nodulin 26 expression results in altered filament growth remains undetermined. The vacuole of higher plants is a complex organelle that has numerous transport activities including various proton pumps, metabolite transporters, and ion channel activities (Martinoia, 1992). Thus, the vacuole plays a critical role in maintaining ion and metabolite homeostasis in higher plant cells. This role is critical since the solute content of the vacuole controls water uptake by plant cells which generates turgor that drives cell elongation growth [reviewed in Steward (1986)]. The vacuole has recently been shown to contain a water channel protein, the γTIP isoform (Maurel *et al.*, 1993). γTIP is predominantly localized in elongating tissues, and it has been suggested that it could play a role in turgor generation and osmoregulation mediated by the vacuole during cell elongation (Ludevid *et al.*, 1992). In addition, other MIP analogs are induced in plant tissues in response to reduction in turgor resulting from drought stress (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992). Thus, plant members of the MIP family could play a role in osmoregulation, which in turn could affect cell expansion growth.

Recently, it was shown that reconstitution of purified nodulin 26 in planar lipid bilayers results in the formation of ion conducting channels (Weaver *et al.*, 1994). Thus, the expression of nodulin 26 on the tonoplast of transgenic plants might upset ionic or osmotic homeostasis resulting in decreased elongation such as that observed in the anther filaments. If this is case, it is somewhat surprising, considering the expression of nodulin 26 in apical and stem tissues, that cell expan-

sion in vegetative tissues appears to not to be overtly affected. Although the reason for this discrepancy is unclear, it is important to consider that nodulin 26's in vitro activity is affected by transmembrane voltage (Weaver *et al.*, 1994), as well as by calcium-dependent phosphorylation (Weaver *et al.*, 1991; Lee *et al.*, 1995). These factors will likely play a role in controlling nodulin 26 activity in vivo.

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