Expression of Genes Encoding Late Nodulins Characterized by a Putative Signal Peptide and Conserved Cysteine Residues Is Reduced in Ineffective Pea Nodules

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Five nodulin genes, *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466*, with reduced expression in ineffective nodules on the pea (*Pisum sativum*) mutant E135 (*sym13*) were characterized. They encode small polypeptides containing a putative signal peptide and conserved cysteine residues and show homology to the nodulins PsENOD3/14 and PsNOD6. For each gene, multiple bands were detected by genomic Southern analysis. Northern analysis showed that all five genes were expressed exclusively in nodules and that their temporal expression patterns were similar to that of the leghemoglobin (Lb) gene during nodule development. Their transcripts were localized predominantly from the interzone II-III to the distal part of nitrogen-fixing zone III in effective nodules, resembling the Lb gene. However, transcripts in ineffective E135 nodules were localized in narrower regions than those in the effective nodules. These results indicate that these nodulins are abundant in pea nodules and that their successive expression during nodule development is associated with nitrogen-fixing activity.

Legume and *Rhizobium* symbiosis is established by a coordinated expression of genes of both partners. Successful symbiosis involves several complex processes represented by root hair curling, infection thread formation, cortical cell division, nodule initiation, and nitrogen fixation, indicating that a number of plant and bacterial genes participate in the symbiotic association. Plant genes expressed exclusively in nodules, termed nodulin genes (van Kammen 1984), are involved in the development of nodules, and dozens of nodulin genes have been isolated to date. Nodulins are divided into early and late nodulins, depending on the time of appearance during nodule organo-

genesis and development (Nap and Bisseling 1990). Some late nodulins have been identified as leghemoglobin (Lb) (Baulcombe and Verma 1978) and enzymes involved in carbon and nitrogen metabolism such as uricase (Bergmann et al. 1983), glutamine synthetase (Gebhardt et al. 1986), and sucrose synthase (Thummler and Verma 1987). Some are present in the peribacteroid membrane surrounding the microsymbiont, and a major protein component, nodulin 26, appears to be a member of the aquaporin water channel family (Rivers et al. 1997). However, the exact functions of the early nodulins and most of the late nodulins remain unclear.

Bacterial mutants that form ineffective nodules arrested at various stages of nodule development have been used to profile the expression of nodulin genes (Dunn et al. 1988; Govers et al. 1987; Jiménez-Zurdo et al. 2000; Padilla et al. 1991; Sengupta-Gopalan et al. 1986). Those analyses appeared to show a correlation of nodulin gene expression with the different stages of nodule development and provide valuable information concerning the functions of nodulin genes. We previously isolated a set of nodulin genes from pea nodules by a subtraction procedure and compared their expression in effective nodules with their expression in ineffective nodules induced on the pea mutant E135 (*sym13*) (Suganuma et al. 1995). The E135 mutant forms ineffective nodules in which nitrogenase proteins are synthesized, but nitrogen-fixing activity is lacking (Suganuma et al. 1998). In this type of ineffective nodule, all the nodulin genes isolated are expressed, but the expression of some is considerably reduced, suggesting that their functions are related to nitrogen-fixing activity.

One of the nodulin genes with reduced expression in ineffective E135 nodules was *PsN5*, a Lb gene (Suganuma et al. 1995). Further characterization of *PsN5* showed that this type of Lb has a higher oxygen-binding affinity than another type of Lb encoded by *PsN120* and is expressed throughout the central tissue of effective nodules (Kawashima et al. 2001). These results suggested that the *PsN5* type of Lb plays a greater role in nitrogen fixation and oxygen consumption of bacteroids by rapid transfer of oxygen to the bacteroids. In this paper, we describe the primary structures of the remaining nodulin genes, *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466*, with reduced expression in ineffective E135 nodules and their temporal and spatial expression patterns during nodule development. Possible functions of these nodulin genes are discussed with respect to nitrogen-fixing activity.

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Nucleotide sequence data reported are available in the Genbank, EMBL, and DDBJ databases under the accession numbers AB059549 (*PsN1*), AB059550 (*PsN6*), AB059551 (*PsN314*), AB059552 (*PsN335*), and AB059553 (*PsN466*).

Table 1. Identification and homology of pea nodulin genes isolated by a subtraction procedure

Clone	Identification	Homology
Clones expressed normally in E135 nodules		
PsN2	Early nodulin ENOD2	
PsN3(89)	Root nodule lectin PsNlec1	
PsN8		<i>Vicia faba</i> late nodulin Nvf-28
PsN12		<i>V. faba</i> glycine-rich protein VfNOD-GRP1
PsN35	Early nodulin ENOD14	
PsN120	Leghemoglobin	
PsN122		<i>V. faba</i> early nodulin VfENOD-GRP5
PsN156	Early nodulin ENOD3	
PsN374	Early nodulin ENOD7	
Clones with reduced expression in E135 nodules		
PsN1		<i>Pisum sativum</i> nodulin ENOD3/14, NOD6
PsN5 (161)	Leghemoglobin	
PsN6(10)		<i>P. sativum</i> nodulin ENOD3/14, NOD6
PsN314		<i>P. sativum</i> nodulin ENOD3/14, NOD6
PsN335		<i>P. sativum</i> nodulin ENOD3/14, NOD6
PsN466		<i>P. sativum</i> nodulin ENOD3/14, NOD6

PsENOD3	MAKILKFVFAII LFFSLFLLSMEA↓EP-----LLPCETDGDC
PsENOD14	SLKFVYAI I LLLSLFLLSMG↓NIP-----LVPCTDDDC
PsNOD6	MAKILKCVFVYAI I LVFFLLI AENVHG↓----AKVKCKKNGDC
PsN1	MAKFLNIVHFMILFISVFFIERNIC↓I-----NVECETDFEC
PsN6	MASILKLYVVFIFLFFFVASNVDG↓RQ-----ACVHDREC
PsN314	MAQIQVFVYTCI I LLSTLLVAYS↓EPI P-----CETDKDC
PsN335	MAEILKLFYI WFFYVLLFVTTSHA↓VER--YKLLPYECEVEDDC
PsN466	MAKTLIFVY-AFLFSLFLLKEADA↓-----QISCTKDKDC
VfNOD-CCP1	MAKFLNIVHFMILLISVFFIERNMS↓T-----IILCKTDADC
VfNOD-CCP2	MAKTLFVY-AFLFSLFLLSKETDA↓-----QISCTKDKDC
VfNOD-CCP3	MTKSLKLCYAI VLFISLFLIVKKIDG↓EPNLMTG YIKCKVDEDC
VfNOD-CCP4	MAKNTKFVYAI VLFSSLFLLSIEA↓NT-----GLRCEIDEDC
VfNOD-CCP5	MTTFIKFVHVMIVFLSLFLVIMNVSA↓S---E--RRTCFTPADC

* *

PsENOD3	PLKPIIETTPMISLHYMCIDKECVLFREVLQTP-----	69
PsENOD14	PMEMSI PSIPNKLLFFMCWEKECVYRRW-----	61
PsNOD6	PKLPHMFP-----I IYRCYQECTLVRVLDSE-----	65
PsN1	QETFPNINK--S-HIVKCIDNLCOFLGKKEVG VNT-----	68
PsN6	KCPGHPVS-----RCVKGFCRCYT FPAIPSFKGMNKY	66
PsN314	PWEIFPL-----VMKCINKFCEYKIVL-----	56
PsN335	PRYIHHP-----QIMKCINLFCRIVYQD-----	64
PsN466	PDISHLK-----LIFKCI NEICEAVKPDAE-----	61
VfNOD-CCP1	IKKDLN-----DWKCIENQCKFVKVAPAFGGTE----	65
VfNOD-CCP2	PDISHLK-----LIFKCI NNICEAVKPDAAE-----	62
VfNOD-CCP3	PTLLVMS-----AYKCIDNLCKLFRLPF-----	66
VfNOD-CCP4	PRDVTILEK--LSYIYKCFDKECIVTEAPF-----	64
VfNOD-CCP5	PTSDCEP-----PSRPFC AFKYCICG-----	59

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Fig. 1. Alignment of the deduced amino acid sequences of *PsN1*, *PsN6*, *PsN314*, *PsN335*, *PsN466*, the early nodulins PsNENOD3 (P25225) and PsENOD14 (P26415), the late nodulin PsNOD6 (S43336), and *Vicia faba* cysteine cluster proteins, CCP1 (AJ243462), CCP2 (AJ243463), CCP3 (AJ243464), CCP4 (AJ243465), and CCP5 (AJ243466). Multiple sequence alignment was carried out using the 'CLUSTAL W' program. Cleavage sites for the putative signal peptides marked by vertical arrows were predicted using the 'SignalP' program. Conserved cysteine residues are marked by asterisks.

RESULTS

Primary structure

of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466*.

The nucleotide sequences of nodulin clones that had been isolated by a subtraction procedure (Suganuma et al. 1995) were determined. Previously, *PsN2* was identified as *ENOD2*, and *PsN5* and *PsN120* were identified as distinct types of Lb genes (Suganuma et al. 1995; Kawashima et al. 2001). In the present study, a BLAST search (Altschul et al. 1997) revealed that *PsN3*, *PsN35*, *PsN156*, and *PsN374* were identified as *PsNlec1* (Kardailsky et al. 1996), *ENOD14*, *ENOD3* (Scheres et al. 1990), and *ENOD7* (Kozik et al. 1996), respectively (Table 1). *PsN89* was identical to *PsN3*, and *PsN161* was identical to *PsN5*. Furthermore, *PsN8*, *PsN12*, and *PsN122* were homologous to *Nvf-28* (Küster et al. 1994), *VfNOD-GRP1*, and *VfENOD-GRP5* (Schröder et al. 1997), respectively. However, five nodulin clones, *PsN1*, *PsN6* (identical to *PsN10*), *PsN314*, *PsN335*, and *PsN466*, with reduced expression in ineffective E135 nodules compared with their expression in effective nodules, were so short in sequence that significant homology to any genes was not detected by BLAST search. Thus, these five cDNA clones containing complete protein-coding regions were re-screened from a nodule cDNA library or prepared by 5' RACE reactions.

Sequence analysis of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* cDNAs indicated that they all encode small polypeptides characterized by the presence of a putative signal peptide at their N-termini and two pairs of cysteine clusters with common structures of Cys-X5-Cys and Cys-X4-Cys (Fig. 1). The same structures were previously found for pea early nodulin

PsENOD3/14 (Scheres et al. 1990), late nodulin *PsNOD6* (Kardailsky et al. 1993), and *Vicia faba* late nodulins designated as cysteine cluster proteins (CCPs) (Frühling et al. 2000). Thus, we postulate that these nodulins belong to gene families with closely related functions. However, sequence similarities of these nodulins other than signal peptides and cysteine clusters were low relative to each other, except for the case of *PsN466* versus *VfNOD-CCP2*. Deduced amino acid sequences of *PsN466* and *VfNOD-CCP2* showed 91.9% identity, whereas sequence identities among the others ranged from 16.4 to 53.6%.

Southern blot analysis of pea genomic DNA with cDNA probes for these five nodulins gave multiple bands (Fig. 2). Re-screening of a nodule cDNA library and 5' RACE screening revealed the presence of two to four distinct mRNA species with a few base pair changes for *PsN1*, *PsN314*, *PsN335*, and *PsN466* in nodules (data not shown). These results strongly suggest that each of these nodulins forms small multigene families.

Temporal and spatial expression patterns.

Transcripts of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* were detected only in nodules (Fig. 3). Their temporal expression patterns were essentially similar to those of the Lb gene (Fig. 4). *PsN6* transcripts appeared most abundantly at 12 days but, following an increase of the amounts of the transcripts, was not as remarkable as those of the Lb gene, *PsN1*, *PsN314*, and *PsN335*. On the other hand, the expression of *PsN466* in 12-day-old nodules was hardly detectable. These results showed that *PsN6* is expressed concomitantly with, or slightly earlier than, the Lb gene, *PsN1*, *PsN314*, and *PsN335*, while *PsN466* appears later than those genes.

Fig. 2. Genomic Southern blot analysis of genes for *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466*. Genomic DNA was isolated from leaves of 3-week-old cv. Sparkle plants. Genomic DNA (10 µg) was restricted with *Bam*HI (B), *Eco*RI (E), and *Hind*III (H), fractionated on a 0.8% (wt/vol) agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled probes.

Transcripts of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* were localized predominantly from the interzone II-III to the distal part of nitrogen-fixing zone III in the effective nodules, resembling the *Lb* gene (Fig. 5). Furthermore, consistent with the results of the temporal expression patterns, transcripts of *PsN6* were also detected in the pre-fixing zone II, and the beginning of expression of *PsN466* was more proximal compared with those of the *Lb* gene, *PsN1*, *PsN314*, and *PsN335*. However, in ineffective E135 nodules, expression regions were narrower than were those in the effective nodules (Fig. 6). In particular, hybridization signals with *PsN466* were very faint. In contrast, the expression of *ENOD12*, *ENOD14*, and *Lb* genes in the ineffective nodules was almost identical to that in the effective nodules. Transcripts of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466*, as well as those of the *Lb* gene, in both effective and ineffective nodules were detectable in the infected cells but not in the uninfected cells (Fig. 7). In Figure 7, only the results of *PsN1* are shown because results of the remaining genes were identical to those of *PsN1*.

DISCUSSION

Fifteen pea nodulin clones that had been isolated by a subtraction procedure were identified (Table 1). Of these clones, expression of six genes was considerably reduced in ineffective E135 nodules compared with their expression in effective cv. Sparkle nodules (Suganuma et al. 1995). Nodules induced on the pea mutant E135 lack nitrogenase activity, whereas organogenesis and morphology of the E135 nodules are the same as those of cv. Sparkle nodules (Kneen et al. 1990; Suganuma et al. 1998). Thus, differences in the expression of these nodulin genes between effective nodules and ineffective E135 nodules appear to represent whether functions of these genes are related to nitrogenase activity. Early nodulins are predicted to be involved in rhizobial infection or nodule initiation (Nap and

Bisseling 1990). Expression of a number of early nodulin genes such as *ENOD2*, *ENOD3*, *ENOD7*, *ENOD14*, and homolog *VfENOD-GRP5* were not affected in ineffective E135 nodules. Late nodulins, *PsNLEC1* and *VfNOD-GRP1*, which are proposed to be components of symbiosomes and of plant cell walls respectively, (Kardailsky et al. 1996; Schröder et al. 1997) were also expressed in ineffective E135 nodules at levels comparable to levels in effective nodules. Since ineffective E135 nodules are morphologically similar to the effective nodules in the early stages of nodule development (Kneen et al. 1990), it is not surprising that nodulins potentially involved in nodule organogenesis and structure are expressed normally in the ineffective nodules. Kardailsky and associates (1996) also showed that the spatial expression pattern of *PsNlec1* in ineffective E135 nodules is similar to that in effective nodules. The late Nvf-28 nodulin is characterized by two types of amino acid repeats flanked by unique amino acid sequence termini, but its function is unclear (Küster et al. 1994). Our results suggest that such nodulins are not associated with nitrogenase activity.

In contrast, roles of nodulin genes whose expression is reduced in ineffective E135 nodules are presumed to be closely associated with nitrogen-fixing activity. Although the expression of six of the nodulin genes is reduced in ineffective E135

Fig. 3. Northern blot analysis of mRNA for *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* in the total RNA isolated from each tissue of cv. Sparkle plants. Nodules, roots, stems, and leaves were harvested from 5-week-old plants, and flowers and pods were harvested from 6-week-old plants. Cotyledons were obtained from 1-week-old seedlings. Each sample of total RNA (10 µg) was subjected to electrophoresis on a 1.25% (wt/vol) agarose gel that contained formaldehyde, transferred to a nylon membrane, and hybridized with radiolabeled probes. The rRNA samples stained with ethidium bromide (EtBr) are shown as controls.

Fig. 4. Northern blot analysis of mRNA for *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* in the total RNA isolated from uninoculated roots and effective cv. Sparkle nodules harvested at different days after sowing. For reference, the *ENOD2* was used as the early nodulin gene and the leghemoglobin (*Lb*) as the late nodulin gene, respectively. Each sample of total RNA (10 µg) was subjected to electrophoresis on a 1.25% (wt/vol) agarose gel that contained formaldehyde, transferred to a nylon membrane, and hybridized with radiolabeled probes. The rRNA samples stained with ethidium bromide (EtBr) are shown as controls.

nodules, it was found that they belong to two groups of nodulin genes (Table 1). One, *PsN5*, is the Lb gene, which has a higher oxygen-binding affinity and is expressed in whole central tissue of the effective nodules (Kawashima et al. 2001). All the others were predicted to encode small polypeptides that contain a putative signal peptide and two pairs of cysteine clusters, such as Cys-X₅-Cys and Cys-X₄-Cys (Fig. 1). They were confirmed to be genuine nodulin genes (Fig. 3). The predicted proteins of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* are structurally homologous to early nodulin ENOD3/14 (Scheres et al. 1990), but according to their temporal and spatial expression patterns (Figs. 4 and 5), they are categorized as late nodulin

genes along with another homologous gene, *PsNOD6* (Kardailsky et al. 1993). Govers and associates (1987) used differential hybridization to screen several nodulin cDNA clones from pea nodules. In addition to *PsNOD6*, they isolated *PsNOD10* and *PsNOD14*, whose in vitro translation products are estimated to be small polypeptides similar to *PsNOD6*. Therefore, it is possible that both *PsNOD10* and *PsNOD14* belong to the same group, although their primary structures remain unpublished. They further showed that mRNA levels of those three genes are decreased in the ineffective nodules induced by *Rhizobium* mutants. In the present study, we could not isolate the gene corresponding to *PsNOD6*, though it is

Fig. 5. In situ localization of mRNA for *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* in effective cv. Sparkle nodules. Nodules were harvested from 4-week-old plants. *ENOD12*, *ENOD14*, and leghemoglobin (Lb) were used for reference. Longitudinal sections (10 μm) through the nodules were hybridized with digoxigenin-labeled antisense probes. Hybridization signals are visible as purple or blue. No significant signals were detected by any sense probe (data not shown). The bar represents 500 μm, and all micrographs were taken at the same magnification.

likely that the expression of *PsNOD6* will be reduced in ineffective E135 nodules. Furthermore, *PsNOD10* or *PsNOD14* might be identical to one of the nodulin genes *PsN1*, *PsN6*, *PsN314*, *PsN335*, or *PsN466*.

Genomic Southern analysis of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* showed multiple bands (Fig. 3). Since we isolated incomplete cDNA clones containing a few base pair changes for *PsN1*, *PsN314*, *PsN335*, and *PsN466* in this study (data not shown), each of the multiple genes is thought to be actually expressed. Therefore, it is probable that nodulins characterized by a putative signal peptide and conserved cysteine residues are abundant in pea nodules. Including the early nodulin genes *ENOD3* and *ENOD14*, at least 15 genes encod-

ing this class of nodulin are predicted to be present in the pea genome. Recently, Frühling and associates (2000) isolated five late nodulin genes encoding CCPs that show similar features in *V. faba* (Fig. 1). In addition, a similar nodulin gene in *Galega orientalis* has been submitted to the DNA database (accession number AJ133497). These data indicate that this class of nodulins is generally present in the nodules. It is notable in studying the origin of this type of nodulin gene that these nodulins are structurally homologous, but similarities of entire amino acid sequences are relatively low (Fig. 1). This contrasts with the *Lb* genes that form a large gene family.

Transcripts of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* were restricted to the infected cell type (Fig. 7), indicating that

Fig. 6. In situ localization of mRNA for *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* in ineffective E135 nodules. Nodules were harvested from 4-week-old plants. *ENOD12*, *ENOD14*, and leghemoglobin (*Lb*) were used for reference. Longitudinal sections (10 μ m) through the nodules were hybridized with digoxigenin-labeled antisense probes. Hybridization signals are visible as purple or blue. No significant signals were detected by any sense probe (data not shown). The bar represents 500 μ m, and all micrographs were taken at the same magnification.

rhizobial invasion is a prerequisite for induction of their expression. Furthermore, their transcripts were detected predominantly from the interzone II-III to the distal part of nitrogen-fixing zone III (Fig. 5), consistent with that of the late nodulin gene *PsNOD6* (Kardailsky et al. 1993). However, *PsN6* expressed slightly earlier and *PsN466* later on the basis of temporal and spatial expression patterns (Figs. 4 and 5). These results suggest that they are differentially regulated. Fröhling and associates (2000) examined the spatial expression patterns of five late nodulin genes encoding CCPs by tissue print hybridizations. They also showed that the distribution patterns of their transcripts are varied, suggesting that their regulation involves different mechanisms.

In ineffective E135 nodules, transcripts of *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* were detected in narrower regions compared with transcripts in effective nodules (Figs. 5 and 6). Therefore, reductions in expression of these nodulin genes in ineffective E135 nodules by northern analysis (Suganuma et al. 1995) are ascribed to the limited distribution of their transcripts. These results are consistent with the case of Lb gene *PsN5*, although reductions in the expression regions of the five nodulin genes in ineffective nodules were not as remarkable as that of *PsN5* (Kawashima et al. 2001). It is likely that the expression of these late nodulin genes is induced without nitrogenase activity, but nitrogenase activity is required for successive expression of their nodulin genes during nodule development.

The problem is to determine the function of this class of nodulins and to identify the target of a putative signal peptide. The expression of early nodulin genes *PsENOD3* and *PsENOD14* is not affected in ineffective E135 nodules, whereas the expression of late nodulin genes *PsN1*, *PsN6*, *PsN314*, *PsN335*, and *PsN466* is reduced (Table 1 and Fig. 6). This may indicate that they are functionally different depending on the time of appearance during nodule development, although they contain the common structures of two cysteine clusters. The conserved cysteine residues may bind to a metal ion. Scheres and associates (1990) assumed that they have a role in the transport of metals over the peribacteroid membrane to the bacteroid, because the bacteroids require molybdenum and iron for the synthesis of nitrogenase. This might explain findings that the transcripts of late nodulin genes were localized from the interzone II-III and the distal part of nitrogen-fixing zone III, where nitrogen-fixing activity begins to appear, and that their expression regions were restricted to narrower cell layers in ineffective E135 nodules (Figs. 5 and 6). In addition, a large amount of iron is required for Lb. In ineffective E135 nodules, the levels of heme were considerably lower than those in effective nodules (Suganuma et al. 1995) while the levels of iron in ineffective nodules were similar to those in effective nodules (N. Suganuma, unpublished data). Moreover, the spatial expression patterns of those late nodulin genes resembled those of the Lb gene in effective nodules (Fig. 5). These results may suggest that this class of late nodulins is concerned with transport of iron for heme of Lb. Otherwise, there is a possibility that the formation of disulfide bonds by this class of nodulins may be subject to redox control in nodule tissue. Moran and associates (2000) showed that legume nodules contain large amounts of the thiol tripeptides glutathione and homogluthathione and that they play critical roles for nodule functioning. In contrast, the transcripts of *PsENOD3/14* are detected in the pre-fixing zone II of pea nodules (Scheres et al. 1990) and their expression in ineffective E135 nodules was comparable to that in the effective nodules (Figs. 5 and 6) (Suganuma et al. 1995). It might be possible that these early nodulins are related mainly to rhizobial infection into nodule cells or proliferation of symbiosomes and that the cysteine resi-

dues act to form disulfide bridges after secretion for certain components.

Although several nodulins proposed to code for peribacteroid membrane proteins have been isolated from soybean nodules, it is not known how proteins are targeted to the peribacteroid membrane, and a signal sequence specific to the peribacteroid membrane has not been identified (Whitehead and Day 1997). Significant similarities in the putative signal sequences are not found between peribacteroid membrane nodulins and nodulins containing conserved cysteine clusters shown in Figure 1. Identification of the target of their putative signal peptides would help greatly for elucidating the functions of these nodulins.

MATERIALS AND METHODS

Plant materials.

The seeds of the parental pea (*Pisum sativum* L. cv. Sparkle) and the Fix⁻ mutant E135 (*sym13*) derived from it (Kneen et al. 1990) were surface-sterilized and inoculated with *Rhizobium leguminosarum* bv. *viciae* strain 128C53. The plants were grown in vermiculite with a nitrogen-free nutrient solution in a greenhouse under natural daylight, as described previously (Suganuma et al. 1993).

cDNA cloning and sequence analysis.

cDNA clones that had been isolated by a subtractive hybridization procedure (Suganuma et al. 1995) did not have complete protein-coding regions. Therefore, recombinant phages derived

Fig. 7. In situ localization of mRNA for *PsN1* in the central part of effective cv. Sparkle nodules and ineffective E135 nodules. Nodules were harvested from 4-week-old plants. Leghemoglobin (Lb) were used for reference. Longitudinal sections (10 μ m) through the nodules were hybridized with digoxigenin-labeled antisense probes. Hybridization signals are visible as purple or blue. The bar represents 100 μ m and all micrographs were taken at the same magnification. IC = infected cell; UC = uninfected cell.

from a λ gt10 cDNA library constructed from poly(A)⁺ RNA of nodules of 5-week-old cv. Sparkle plants were rescreened with each cDNA insert as a probe. Since the cDNA clones containing the entire coding region for *PsN1* and *PsN335* were not obtained by rescreening, the 5' RACE system (Gibco-BRL, Rockville, MD, U.S.A.) was used to obtain their 5'-upstream sequences. Isolated cDNA inserts from positive recombinants or amplified cDNAs were subcloned into pBluescript II SK(+) (Stratagene, La Jolla, CA, U.S.A.) and their nucleotide sequences were determined with an automatic ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Nucleotide and deduced amino acid sequences were analyzed with the GeneWorks program (Intelligenetics, Mountain View, CA, U.S.A.). Multiple sequence alignment was carried out using the CLUSTAL W program (Thompson et al. 1994) of the DNA Data Bank of Japan (Mishima, Japan). Predictions of signal peptides and their cleavage sites were performed with the SignalP program (Nielsen et al. 1997).

Southern and northern blot analyses.

Genomic DNA was isolated from the leaves of 3-week-old cv. Sparkle plants according to the method of Murray and Thompson (1980). Genomic DNA was digested to completion with *Bam*HI, *Eco*RI, and *Hind*III. Total RNAs were isolated from various tissues of cv. Sparkle plants or nodules harvested from cv. Sparkle plants at different days after inoculation by phenol extraction and LiCl precipitation (Suganuma et al. 1995). DNA fragments were separated on a 0.8% (wt/vol) agarose gel, and total RNAs were subjected to electrophoresis on a 1.25% (wt/vol) agarose gel that contained formaldehyde and transferred to a Hybond-N membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) according to standard procedures (Sambrook et al. 1989). DNA probes were prepared from each cDNA insert with ³²P-dCTP and a *Bca*BEST Labeling Kit (TaKaRa, Kyoto, Japan). *PsN2* (*ENOD2*; Suganuma et al. 1995) and *PsLb120-1* (Kawashima et al. 2001) were used for reference as the early and late nodulin genes. Hybridization and washing were done as described previously (Suganuma et al. 1997).

In situ hybridization.

In situ hybridization was performed as described by Kouchi and Hata (1993). RNA probes were prepared from linearized plasmids with digoxigenin-11-UTP (Roche Diagnostics, Basel, Switzerland), and the hybridization signals were detected by antidigoxigenin-alkaline phosphatase conjugate with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (Roche Diagnostics, Basel, Switzerland). *ENOD12* cDNA was previously prepared by amplification by polymerase chain reaction (Suganuma et al. 1995). *PsN35* (*ENOD14*) and *PsLb120-1* were also used for reference.

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