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Short communication

The pea early nodulin gene *PsENOD7* maps in the region of linkage group I containing *sym2* and leghaemoglobin

Alexander Kozik[1], Martha Matvienko[1], Ben Scheres[1,5], V.G. Paruvangada[4], Ton Bisseling[1,*], Ab van Kammen[1], T.H. Noel Ellis[2], Tom LaRue[3] and Norman Weeden[4]

[1] *Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, Netherlands (*author for correspondence);* [2] *John Innes Institute, John Innes Centre, Norwich Science Park, Colney Lane, Norwich, NR4 7UH, UK;* [3] *Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, USA;* [4] *Department of Horticultural Sciences, Cornell University, Geneva, NY 14456, USA;* [5] *Present address: Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, Netherlands*

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Abstract

The early nodulin gene, *PsENOD7*, is expressed in pea root nodules induced by *Rhizobium leguminosarum* bv. *viciae*, but not in other plant organs. *In situ* hybridization showed that this gene is transcribed during nodule maturation in the infected cells of the proximal part of the prefixation zone II. At the transition of zone II into interzone II–III, the level of *PsENOD7* mRNA drops markedly. *PsENOD7* has no significant homology to other genes. RFLP mapping studies have shown that *PsENOD7* is located in linkage group I between the leghaemoglobin genes and *sym2*.

Rhizobium leguminosarum bv. *viciae* induces the formation of nitrogen fixing root nodules on the roots of *Pisum sativum* (pea). By mutagenesis and genetic studies several plant genes essential for normal nodule development have been identified and these genes have been named *sym* genes. In pea about 30 different *sym* genes have been described [7, 18, 19, 26]. The *sym* genes are distributed randomly on the seven linkage groups of pea [19], but several *sym* genes, namely *sym2*, *sym5*, *sym19* and *nod3*, are clustered on linkage group I, near the major leghaemoglobin (*Lb*) locus [32, 38].

The different stages of legume nodule development are accompanied by the expression of plant genes, the so-called nodulin genes. These genes, that are only expressed during nodule development, have been divided into early and late nodulin genes; the early nodulin genes are expressed before the bacteria start to fix nitrogen, whereas

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X93172.

the late nodulin genes are induced around the start of nitrogen fixation [24].

Nodulin genes have been identified in several legumes such as soybean, pea, *Medicago*, *Phaseolus*, *Sesbania*, *Vicia* and lupon (for reviews see [11, 28]). In pea six early nodulin genes have been described, such as *PsENOD12* [29] and *PsENOD40* [22], and several late nodulin genes, such as glutamine synthetase (*GS*) [33], leghaemoglobin (*Lb*) [23] and *PsNOD6* [16].

At present, it is unknown whether some of the pea *sym* genes encode nodulins. To answer the latter question it is essential to determine the positions of both *sym* and nodulin genes on genetic map and to check whether their position coincides.

In this paper, we describe the molecular characterisation of the early nodulin cDNA clone pPsENOD7, the *in situ* expression pattern of the corresponding gene, as well as the position of the gene on the genetic map.

Isolation of pPsENOD7

A λgt11 cDNA library, prepared from *Pisum sativum* cv. Sparkle root nodule RNA, was kindly provided by G. Coruzzi [33] and seven early nodulin cDNA clones were isolated by differential screening [30]. Previously, we have described the characterization of six clones, namely pPsENOD2, pPsENOD3, pPsENOD5, pPsENOD12, pPsENOD14 and pPsENOD40 [22, 29, 30, 34]. Here, we present the characterization of pPsENOD7.

PsENOD7 *is expressed only in nodules*

Southern blot analyses revealed that the insert of pPsENOD7 hybridized to a single fragment in *Eco*RI (1 kb) or *Hin*dIII (8 kb) digested DNA from pea cv. Rondo (data not shown). These data indicate that PsENOD7 is encoded by a single gene.

We studied the expression of *PsENOD7* by northern blot analysis of RNA from uninfected roots, from roots 4 and 8 days after sowing and inoculation with *R. leguminosarum* bv. *vicae* strain 248 and from 15-day old nodules. *PsENOD7* mRNA had a length of 500 bp (Fig. 1). *PsENOD7* mRNA was not detectable 4 days after inoculation, but it was present at a low level after 8 days and it accumulated to a markedly higher level in 15-day old nodules. The transcript was absent in shoots, hypocotyls, epicotyls, flowers, leaves, pods, cotyledons, and uninfected roots. Furthermore, the gene was not induced in pea roots 12, 60 and 84 h after inoculation with the fungal pathogen *Fusarium oxyporum* (Fig. 1). Hence, *PsENOD7* appears to be a true nodulin gene [35].

In our preliminary experiments we could not detect induction of *PsENOD7* mRNA expression with purified Nod factors (data not shown), which is consistent with the fact that 4 days after inoculation with *R. leguminosarum* bv. *viciae ENOD7* expression is not detected (Fig. 1).

In situ *localization of* PsENOD7 *mRNA*

Pea forms nodules with an indeterminate growth pattern like most other temperate legumes. Thus a gradient of developmental stages is present from apex to root attachment point and consequently, the nodule central tissue can be divided in zones representing subsequent stages of development; zone I is the apical meristem, followed by prefixation zone II, interzone II-III and fixation zone III [12, 36]. At the transition of interzone II-III into fixation zone, amyloplast accumulation at the periphery of infected cells suddenly starts [12, 36].

Longitudinal sections of 14-day old pea nodules were hybridized with ^{35}S labelled antisense as well as sense *PsENOD7* RNAs. The sense probe gave no signal above background (result not shown), whereas the antisense probe hybridised with RNA present in infected cells (Fig. 2a, b). *PsENOD7* mRNA was first detectable in the proximal part of the prefixation zone II and reached its maximal level at the transition of the prefixation zone into interzone. At this transition the level of *PsENOD7* transcript

suddenly dropped to a markedly lower level (Fig. 2c, d).

It has been shown that at the transition of prefixation zone II into interzone II-III the expression level of several bacterial and plant genes rapidly changes. For example, the expression of *ropA* of *Rhizobium* is switched off, whereas the expression of the rhizobial *nif* genes is induced at this transition [3, 6]. So the expression level of *PsENOD7* markedly drops when the bacteria acquire the ability to fix nitrogen. Together with the decrease of the expression of the *PsENOD7*, the expression of some other pea early nodulin genes such as *PsENOD5* and *PsENOD3* is down-regulated, whereas the late nodulin gene *PsNOD6* [16] and the alfalfa leghaemoglobin genes are induced at this stage of development [5]. Hence, the down regulation of *PsENOD7* at the prefixation zone/interzone transition provides additional evidence that at this transition a dramatic and rapid change in nodule development takes place.

Sequence of pPsENOD7

The insert of pPsENOD7 was sequenced using the dideoxy chain termination method with an automatic sequencer (Applied Biosystems model 373A). The cDNA insert of pPsENOD7 was 432 bp in length including a poly(A) tail at the 3′ end, while the *PsENOD7* mRNA had a size of about 500 bp [see above]. Therefore, the missing 5′ part of *PsENOD7* RNA was cloned. Using 5′ RACE [13] with the modifications by Kardailsky [17], we obtained a clone of 184 bp containing 108 bp of the 5′ end of the insert of pPsENOD7 and 76 bp of the missing 5′ end. The *PsENOD7* cDNA sequence contained a single large open reading frame with the first ATG codon at position 24. The putative ENOD7 polypeptide is 115 amino acids long (Fig. 3) with a size of 12 kDa ENOD7 is a hydrophilic protein with a hydrophobic domain at the N-terminal end, which may be part of a putative signal peptide [37]. This suggests that ENOD7 is transported across a membrane and, hence, it might be a protein lo-

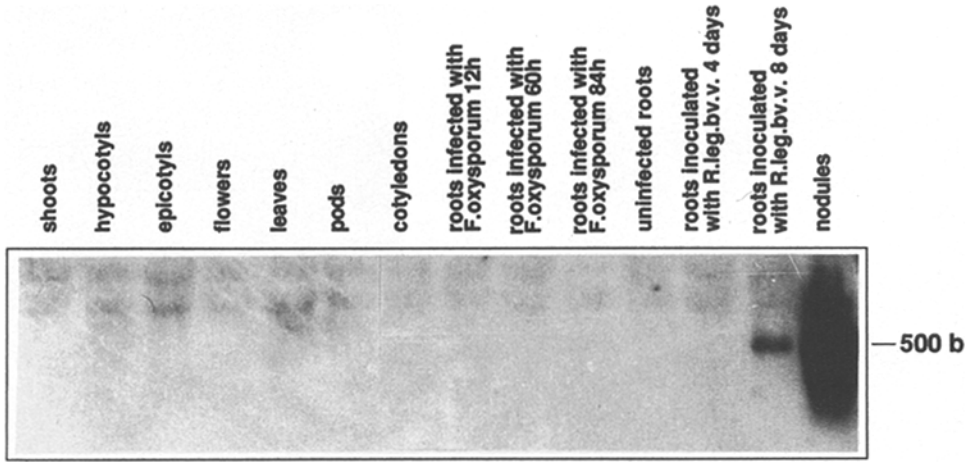

Fig. 1. Expression of *PsENOD7* in different plant organs. Pea (*Pisum sativum* cv. Sparkle) plants were cultured and inoculated with *R. leguminosarum* bv. *viciae* strain 248 as described previously [2]. Plant organs were harvested from pea plants at different time points: shoots and cotyledons from 7-day old plants; hypocotyls, epicotyls and roots from 14-day old plants; flowers, leaves and young pods from 45-day old plants. Inoculated roots were harvested 4 and 8 days after inoculation; nodules were harvested 15 days after inoculation. Total RNA was extracted from plant tissues as described previously [14]. *Fusarium oxysporum* mycelium was inoculated in Czapek-dox medium and grown for 2 days at 30 °C. Pea plants were inoculated with this suspension 3 days after sowing. *Fusarium*-infected roots were harvested at 12, 60 and 84 h after inoculation. Northern hybridization showed that the pathogenesis-related gene, chalcone synthase, is induced in the *Fusarium*-infected roots (data not shown).

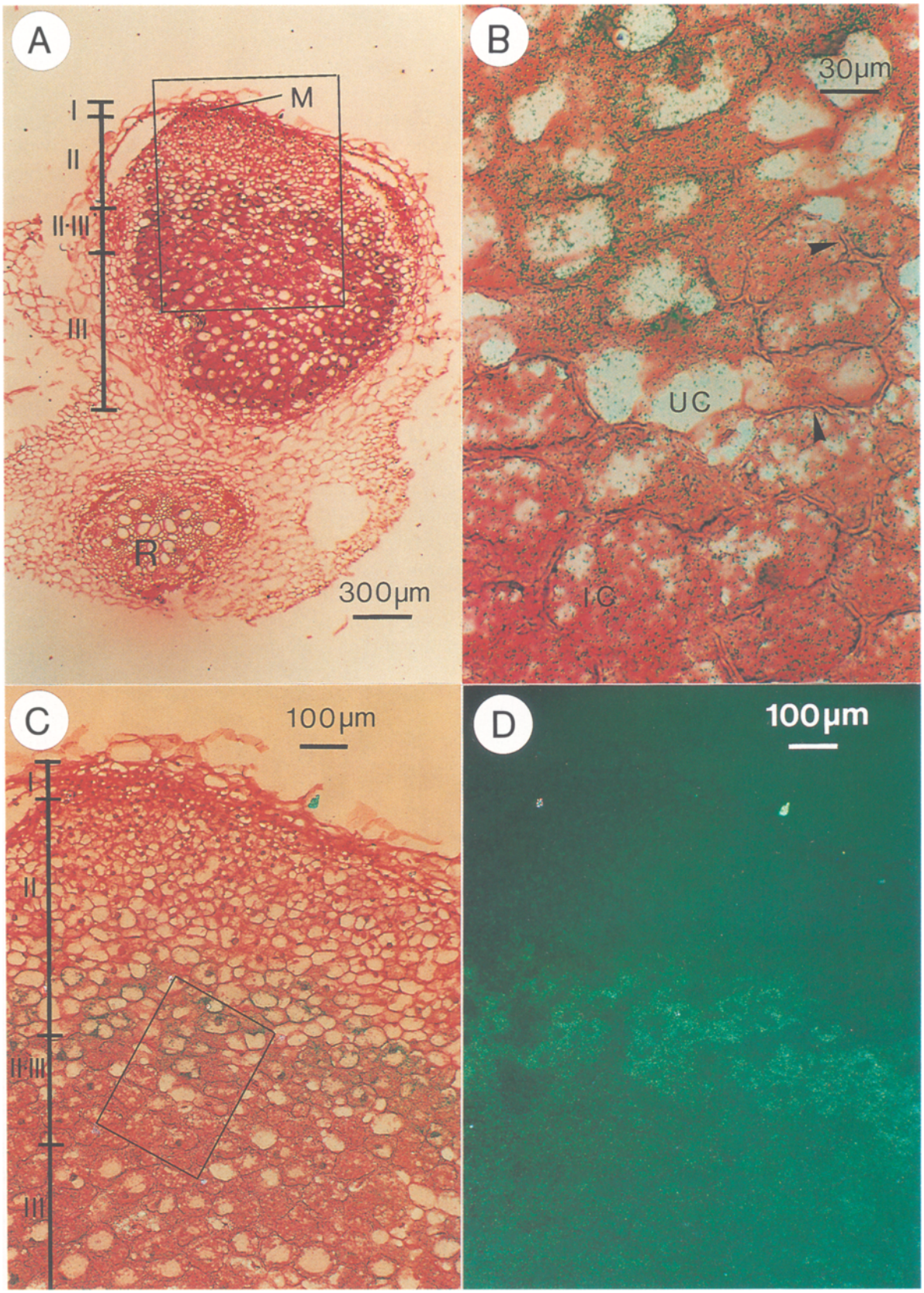
A
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Fig. 3. Nucleotide sequence of the insert of pPsENOD7 and deduced amino acid sequence. Position of the cleavage site of the putative signal peptide is indicated by Δ. The part of the sequence obtained after 5′-RACE is indicated in italics and the sequence present in both pPsENOD7 and the 5′-RACE clone is underlined. Two *PsENOD7*-specific antisense oligonucleotides, and two universal primers (with multiple cloning sites): CTCGAGGATCCGCGGCCGC$(T)_{18}$ and GCTCGAGGATCCGCGGC were used to amplify the 5′ region of *PsENOD7* mRNA. The antisense oligo's used for 5′-RACE are overlined.

cated in the space between rhizobia and the peribacteroid membrane or an extracellular protein. *PsENOD7* has no significant homology to other sequences present in the databases of the National Center of Biotechnology Information (NCBI), National Library of Medicine, NIH (Bethesda, MD). Database searches were performed using the BLAST algorithm [1].

Mapping of PsENOD7

The position of *PsENOD7* on the pea genetic map was determined in order to find out the relation of *PsENOD7* to previously identified *sym* genes. By using the segregating population of cross JI1794 × Slow [39], we showed that *PsENOD7* is closely linked to the major *Lb* locus of linkage group I, in the region where *sym2* is also located [38] (data not shown). *sym2* is the gene of Afghanistan peas which confers resistance to form nodules with *Rhizobium leguminosarum* bv. *viciae* strains lacking the nodulation gene *nodX* [10, 21].

Recently, we have determined the position of *sym2* on the RFLP map of pea constructed by Ellis [8] and shown that it is flanked by the RFLP markers 44 and 267 [20]. We used segregating F2 and F3 (single seed descent from F2) populations

Fig. 2. In situ localization of *PsENOD7* mRNA in a 14-day-old nodule of pea. A. Bright-field picture of a longitudinal section through a pea nodule. I, meristem (M); II, prefixation zone; II-III, interzone; III, fixation zone; R, root. B. A combination of epipolarisation and bright field micrograph of the boxed area in C, showing the decrease in the level of *PsENOD7* mRNA at the transition of prefixation zone into interzone. Green dots are silver grains representing the signal. Amyloplasts in the infected cells are indicated by arrowheads; IC, infected cells; UC, uninfected cells. C. A combination of epipolarization and bright-field micrograph of the part of the nodule indicated in A. D. Epipolarization micrograph of C showing that *PsENOD7* mRNA accumulation starts in the proximal part of prefixation zone, and the level drops markedly at the transition of prefixation zone into interzone (bright-green dots are silver grains representing the signal). The preparation of sections and hybridization conditions are according to a procedure described previously [4, 34].

Table 1. Pairwise data for cross L-4 × 1238 (combined data for F2 and F3 populations).

Pair of markers	Recombination %	LOD
sym2/Lb	5.1 ± 1.7	35.8
sym2/cDNA164	4.0 ± 1.5	39.1
sym2/cDNA44	1.0 ± 0.7	51.6
sym2/cDNA267	6.6 ± 1.9	31.9
sym2/ENOD7	1.7 ± 0.9	47.7
ENOD7/cDNA267	9.1 ± 2.3	26.5
ENOD7/cDNA44	0.7 ± 0.6	53.5
ENOD7/cDNA164	2.2 ± 1.1	46.6
ENOD7/Lb	3.2 ± 1.3	42.5
Lb/cDNA164	1.0 ± 0.7	51.9
Lb/cDNA44	4.0 ± 1.5	39.8
Lb/cDNA267	13.0 ± 2.8	19.6
cDNA164/cDNA44	2.9 ± 1.3	43.5
cDNA164/cDNA267	11.8 ± 2.6	21.5
cDNA44/cDNA267	8.2 ± 2.2	28.6

To position *PsENOD7* on the RFLP map of pea [8] we used the RFLP markers cDNA44, cDNA164, cDNA267 and Lb that are located around the *sym2* locus. Two segregating populations (F2 and F3 of cross L-4 (carrying *sym2*) × NGB1238) were used for mapping, each contains 64 plants. Genomic DNA was isolated from young pea leaves as described previously [25] and digested with *Hin*dIII. Restriction enzyme digestion, gel electrophoresis, Southern blotting and filter hybridization (Hybond-N$^+$ membrane, Amersham) were performed by standard protocols [27]. The RFLP probes were labelled with α-^{32}P dATP using the random priming method [9].

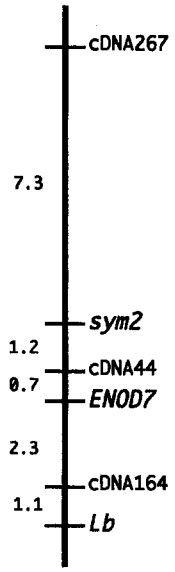

Fig. 4. RFLP map of the *sym2* locus of linkage group I of pea.

of the cross L-4 × NGB1238 [20] to position *PsENOD7* on the RFLP map. Linkage analysis was performed using the program JoinMap, version 1.4 [31]. The results presented in Table 1 and Figure 4, show that *PsENOD7* is located about 2 cM below *sym2* and 3.5 cM above the *Lb* locus. A confirmation of the order of markers in the *sym2* region was obtained by determining the sites of recombination in *Pisum sativum* cv. Rondo lines containing an introgressed *sym2* area of pea cv. Afghanistan [20] (data not shown).

We have observed a single recombination between *PsENOD7* and *sym2* gene among 64 plants of the segregating F2 population of the cross L-4 × NGB1238, in a plant having no Afghanistan *sym2* allele. In the F3 offsprings derived from this plant, we found plants which were homozygous for the *PsENOD7* Afghanistan allele, as shown by RFLP analysis, but lacked the Afghanistan *Sym2* allele (data not shown). This further demonstrates that *PsENOD7* does not coincide with *sym2*, while the low frequency of recombination shows that *PsENOD7* is tightly linked to this locus.

It is striking that two nodulin genes *PsENOD7* and *Lb*, and *sym2* map relatively close to each other. Furthermore, it has been shown that *sym* gene, *nod3*, is also located in the vicinity of *sym2* [32]. The *nod3* mutant has lost the ability to autoregulate nodule number and hence forms markedly more nodules than wild type peas [15]. Although the exact position of *nod3* is still not known, *sym2* and *nod3* are not allelic (Kozik, Temnykh and Weeden, unpuplished results). Furthermore, it is unlikely that *PsENOD7* and *nod3* are allelic, since *PsENOD7* is expressed in the proximal part of the prefixation zone of the central tissue. It is not probable that a gene expressed at this stage of nodule development can control nodule number. Moreover, by northern analysis we did not detect a difference in the level of *PsENOD7* expression in nodules of wild type pea cv. Rondo and *nod3* mutant (data not shown). Therefore, we conclude that *nod3* and *PsENOD7* are different genes and thus the region on linkage

group I harbouring *PsENOD7* contains at least four genes involved in the *Rhizobium*-legume symbiosis; *sym2*, *nod3*, *PsENOD7* and *Lb*.

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