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A Gene That Encodes a Proline-Rich Nodulin with Limited Homology to PsENOD12 Is Expressed in the Invasion Zone of *Rhizobium meliloti*-Induced Alfalfa Root Nodules[1]

Marian Löbler[2] and Ann M. Hirsch*

Department of Biology, University of California, Los Angeles, 405 Hilgard Avenue, Los Angeles, California 90024-1606

To define the early stages of the interaction between *Rhizobium* and host legumes, we have cloned and characterized three early nodulin-encoding sequences from an alfalfa (*Medicago sativa* L.) cDNA library by probing with a fragment of a cDNA clone for PsENOD12, an infection-related nodulin from pea (*Pisum sativum* L.). Although the coding regions of the three clones are 95 to 98% homologous to each other, they are only 43% homologous to the pea clone. However, the putative signal peptide encoded by the alfalfa cDNA clones is 100% homologous to the PsENOD12 signal peptide. The spatial and temporal expression patterns of PsENOD12 and the alfalfa clones were compared. In situ hybridization experiments detected RNA transcripts in the invasion zone of mature nitrogen-fixing nodules, the same site where PsENOD12 mRNAs are found. Transcripts were also found by in situ hybridization in cells of *Rhizobium meliloti exoH* mutant-induced nodules penetrated by infection threads, but northern analysis did not detect transcripts in Inf⁻ (infection thread minus) nodules elicited by *R. meliloti exoB* nodules or in pseudonodules elicited by treatment with the auxin transport inhibitor *N*-1-(naphthyl)phthalamic acid. In addition, the alfalfa gene represented by these cDNA clones exhibited a temporal expression pattern that differed from that of PsENOD12, which is transiently expressed. These data, plus information derived from Southern blot analysis, indicate that we have isolated cDNA clones for a novel early nodulin, which we have designated MsENOD10 (*Medicago sativa* Early Nodulin 10).

Leguminous plants and bacteria of the genus *Rhizobium* associate in a symbiosis in which atmospheric nitrogen is converted into ammonia, a source of nitrogen that can be utilized by the plant. During the establishment of the nitrogen-fixing symbiosis, both partners undergo profound morphological and physiological changes. Many of the bacterial genes involved in the symbiosis have been identified, and the products of their genes have been extensively characterized (for a review, see Long, 1992). The *Rhizobium nod* genes are a well-studied example. When the *nod* genes are induced by plant-derived flavonoids, the bacteria produce Nod factors, substituted glycolipids that promote root hair deformation and cell divisions in the inner cortex of alfalfa and vetch roots (Lerouge et al., 1990; Spaink et al., 1991; Schultze et al., 1992). These signal molecules also elicit the expression of plant genes involved in nodule formation (see refs. in Fisher and Long, 1992; Hirsch, 1992). Further development of the nodule requires the expression of numerous genes, from both the plant and *Rhizobium*, in addition to the *nod* genes.

The plant exerts a great deal of control over nodule formation. However, dissecting the role of the plant, particularly in the early stages of the symbiosis, has been more difficult than elucidating the role of *Rhizobium*. The use of plant mutants to study genes involved in establishing the symbiosis has been limited, and as yet, no correlation exists between a known mutation and the identity of a specific enzyme or structural protein (for reviews, see Rolfe and Gresshoff, 1988; Phillips and Teuber, 1992). Various biochemical and molecular biological methods have been exploited to study nodule-specific proteins (nodulins) and nodule-specific genes. The nodulins were originally identified by analyzing nodule proteins that had remained after immunoprecipitation with antibodies to root proteins (Legocki and Verma, 1980). Additional studies have led to the isolation of a number of nodule-specific genes and cDNAs (Delauney and Verma, 1988) as well as the classification of nodulins into early and late (Govers et al., 1987a). Bisseling and colleagues were the first to identify cDNA clones of the early nodulins, proteins that are synthesized as the nodule develops (Govers et al., 1985, 1987a, 1987b). Expression of early nodulins is one of the plant's earliest responses to *Rhizobium*-produced signal molecules.

Several of the ENOD clones appear to encode (hydroxy)Pro-rich glycoproteins, which are probably components of the plant cell wall (Nap and Bisseling, 1990). ENOD2, the first such ENOD to be characterized, was originally isolated as a cDNA clone (GmENOD2) from a soybean nodule library (Franssen et al., 1987), whereas ENOD12 was first identified in pea (Scheres et al., 1990). Its deduced amino acid sequence shows that PsENOD12, like ENOD2, is a Pro-rich protein. Moreover, PsENOD12 is related to the SrPRP proteins, which are Hyp-rich cell-wall proteins of soybean (Hong et al., 1990). PsENOD12 transcripts are found in

[1] Supported by National Science Foundation Grant DCB 90-21597.

[2] Present address: Institut für Pflanzenbiochemie, Weinberg 3, O 6120 Halle, Germany.

* Corresponding author; fax 1-310-206-5413.

Abbreviations: ENOD, early nodulin; MsENOD, *Medicago sativa* early nodulin; MtENOD, *Medicago truncatula* early nodulin; NPA, *N*-(1-naphthyl)phthalamic acid; PCR, polymerase chain reaction; PsENOD, *Pisum sativum* early nodulin.

several pea cell types including root hairs 24 to 48 h after inoculation with *Rhizobium leguminosarum* bv *viciae*; root cortical cells in advance of and penetrated by infection threads; uninfected cells of the nodule primordium; and cells of the invasion zone of mature pea nodules (Scheres et al., 1990). Because PsENOD12 mRNAs were detected in uninfected cells as well as in cells containing infection threads, Scheres et al. (1990) suggested that PsENOD12 is involved in the infection process, perhaps in preparing the host cells for invasion. Recently, we have shown that PsENOD12 can be induced in the absence of *Rhizobium* as well as infection thread formation. PsENOD12 transcripts have been detected in pea root hairs 48 h after treatment with the auxin transport inhibitor NPA as well as in NPA-induced Afghanistan pea pseudonodules (Scheres et al., 1992).

Pichon et al. (1992) have subsequently identified a gene homologous to PsENOD12 from a genomic library of *Medicago truncatula*, a diploid, autogamous medic that is nodulated by *R. meliloti*. MtENOD12 exhibits spatial and temporal expression patterns in *M. truncatula* that are very similar to those of PsENOD12 in pea. In addition, like PsENOD12, the MtENOD12 gene is expressed in uninfected root cells 3 to 6 h after *Rhizobium* inoculation, before infection threads are evident.

Our goal was to determine whether a gene homologous to the PsENOD12 gene was expressed in alfalfa (*Medicago sativa*) nodules. We wanted to test whether invasion was required for ENOD12 gene expression in alfalfa. We also wanted to find a gene that could serve as an early genetic marker for studies on signal exchange between *R. meliloti* and its host. Alfalfa offers the advantage that it has been well studied with regard to points of nodule arrest following inoculation with various invasion-defective *R. meliloti* mutants, especially the exopolysaccharide (*exo*) mutants (Finan et al., 1985; Leigh et al., 1987; Yang et al., 1992). It also can be transformed using *Agrobacterium*-derived vectors (Deak et al., 1986). We report here on the identification of three alfalfa ENOD cDNA clones that differ significantly in DNA sequence from the PsENOD12 clone used to identify them. We found a number of additional differences between the PsENOD12 clone and the alfalfa ENOD cDNAs, leading us to believe that we have isolated cDNA clones for a novel ENOD gene of *Medicago sativa*, which we have named MsENOD10.

MATERIALS AND METHODS

Plants

Alfalfa (*Medicago sativa* L. cv Iroquois) seeds were sterilized for 30 to 60 min in 95% ethanol followed by full-strength household bleach (2 × 30 min). The seeds were rinsed four times for 15 min in sterile, distilled water. These sterilization conditions did not have any adverse effects on germination or on seedling morphology or viability. Seeds were planted in sterilized perlite, watered with nitrogen-free Jensen's medium (Vincent, 1970), and subsequently inoculated with bacteria (approximately 10^8 cells/mL). Plants were grown at 20°C under a 16-h light/8-h dark regime. Nodules were harvested after 3 weeks, immediately frozen in liquid nitrogen, and stored at −80°C. Pseudonodules were induced on alfalfa seedlings according to Hirsch et al. (1989). Three to 4 weeks after NPA application, pseudonodules were harvested and stored as described above.

In other experiments, sterilized seeds were placed on cheesecloth stretched over a metal screen that had been sterilized prior to planting. The screen was placed in a sterile plastic dish pan with Jensen's medium such that the screen was held 2 cm above the medium. The overhanging edges of the cheesecloth provided contact to the medium so that the seeds did not dry out. The dish pan was covered with Saran Wrap, and plants were grown under the conditions given above. After 3 d, plants were inoculated with wild-type rhizobia. Root tissue was harvested prior to inoculation and at 1, 2, 4, 6, 8, 10, 14, and 18 d postinoculation. Upon harvest, tissue was frozen immediately in liquid nitrogen and stored at −80°C. A clone of *M. sativa* L. cv Regen was used for harvest of tissues other than roots and nodules. Plants of *Medicago truncatula* L. cv Jemalong were also maintained in the greenhouse for harvest of tissues.

Rhizobia

Wild-type *Rhizobium meliloti* strain Rm1021 (Meade et al., 1982) and *exo* mutants Rm5078 (*exoB*; De Vos et al., 1986) and Rm7154 (*exoH*; Leigh et al., 1987) were grown in *Rhizobium* defined medium (Vincent, 1970) at 30°C until an OD_{600} of approximately 0.2 was reached.

Clones and Vectors

We used the bacteriophage λgt11 (Amersham) and the plasmids pUC119 and pBSM13+ (Stratagene) as cloning vectors. Plasmid pPsENOD12 (Scheres et al., 1990) was kindly provided by Ton Bisseling. The alfalfa ENOD2 cDNA pA2ENOD2 (Dickstein et al., 1988) was a gift from Rebecca Dickstein, and the alfalfa leghemoglobin cDNA (MsLb3) was isolated by the authors (Löbler and Hirsch, 1992). A mixed rDNA probe for pea chloroplast and cytosolic rRNA was provided by Wilhelm Gruissem.

Nucleic Acid Isolation and Blotting

RNA was isolated from tissue following the procedure of Goldberg et al. (1981) and stored in either 70% ethanol at −20°C or in TE at −80°C. Poly(A)$^+$ RNA was isolated via oligo(dT) affinity chromatography as described (Maniatis et al., 1982) and stored in 70% ethanol at −20°C.

RNA was separated on a 1% agarose gel in the presence of 6.7% formaldehyde (Maniatis et al., 1982) and blotted onto Nytran membranes following the protocol provided by Schleicher & Schuell. Size comparisons were made to a 0.24- to 9.5-kb RNA ladder (BRL).

Genomic DNA was isolated from *M. truncatula* or alfalfa leaves according to Cone (1989) with minor modifications (Löbler and Hirsch, 1990). Plasmid DNA was prepared according to established procedures (Maniatis et al., 1982). DNA was digested with restriction enzymes (BRL), and the resulting fragments were separated on either a 0.8% or a 1% agarose gel. *Hin*dIII fragments of bacteriophage λ (BRL) were used as size standards. Southern blotting onto Nytran mem-

branes was performed according to the Schleicher & Schuell protocol.

Isolation of Clones

A cDNA library was constructed from poly(A)$^+$ RNA isolated from 21-d-old nodules in the λgt11 vector using the cDNA synthesis kit and the λgt11 cloning kit from Amersham. After amplification, the bacteriophage library was plated on Y1090 cells at a density of 25,000 plaques per agar plate (15 cm in diameter). Plaque lifts on nitrocellulose filters (Schleicher & Schuell) were screened with a radioactively labeled 400-bp *Eco*RI/*Rsa*I fragment of the PsENOD12 cDNA clone (oligolabeling kit, Pharmacia). Hybridization and washes were performed as described (Maniatis et al., 1982). Phage from positive plaques were purified by three more screenings. Additional phage clones were isolated by screening the library with an alfalfa clone obtained after the first screen. Positive bacteriophage clones were amplified by the plate lysis method and purified on a CsCl step gradient, and DNA was isolated using the formamide method (Lech, 1989a, 1989b). Phage DNA inserts were then subcloned into pUC119. The *Eco*RI ends of two PsENOD12-hybridizing inserts of 660 and 509 bp were filled in with the Klenow fragment of DNA polymerase I (BRL) and subcloned into the *Sma*I site of pBSM13+, yielding the clones pBS.E12–0 and pBS.E12–1, respectively.

Sequence Analysis

The DNA sequences were determined by dideoxy sequencing of double-stranded plasmid DNA using a sequencing kit (United States Biochemical) and [α-^{35}S]dATP (New England Nuclear). DNA fragments were separated on a 8% (or 4%) wedge polyacrylamide gel (BRL) in the presence of 7 M urea (Sanger et al., 1977).

For sequence analysis and comparison, we used the University of Wisconsin DNA analysis package (Devereux et al., 1984). For sequence comparison to other known DNA or protein sequences, the following data bases were used: GenBank, EMBL, National Biological Resource Foundation, SwissProt, and the DNA data base of Japan.

PCR

PCR amplification of alfalfa mRNA was performed with the heat-stable reverse transcriptase rTth (Perkin Elmer) primed by a 3′ end-specific primer (^{342}CGTGGTAGCTTCTGAGATGG^{323}) according to the manufacturer's protocol. After reverse transcription at 70°C, the 5′ end-specific primer (^{17}GCTTCCTTTTCTTGTCCTCAC^{38}) was added, and cDNA was amplified in 35 cycles of 95°C for 1 min, 60°C for 1 min, with a final incubation at 60°C for 7 min. Temperature cycles were run on an Ericomp (San Diego, CA) Easy Temperature Cycler. The primer coordinates refer to the pUC.E12–1 cDNA sequence (Fig. 1).

In Situ Hybridization

Nodules were cut from plants, immediately placed in 3.7% formaldehyde, 2.5% acetic acid, 50% ethanol, and fixed for

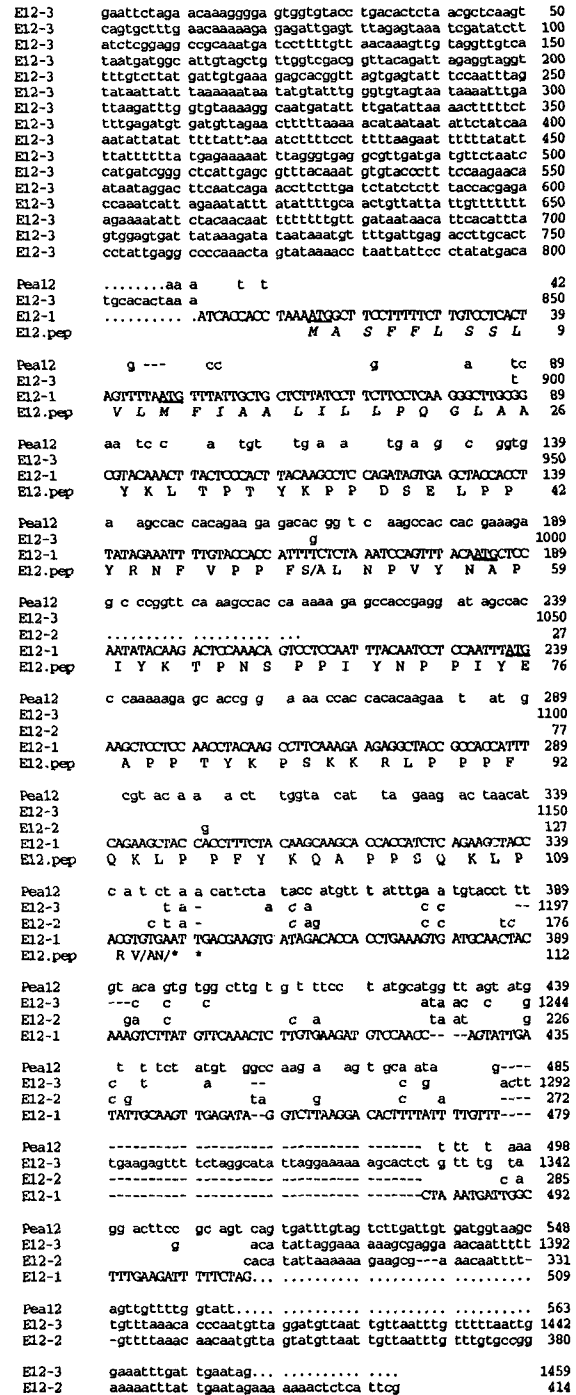

Figure 1. Comparison of the nucleotide sequences of the MsENOD10 cDNA clones (E12–1, E12–2, and E12–3) with the PsENOD12 cDNA (Pea12). Regions of identity are blank, and deviations from the MsENOD10 sequence are written in lowercase letters. All potential start codons are underlined, and the longest open reading frame of E12–1 is translated into the one-letter amino acid code (E12.pep). The putative signal peptide is in italics. Deviations from the nucleotide sequence of E12–1 that lead to different amino acids are added to particular amino acids with slashes (/). Dots indicate regions where no sequence information is available, and dashes indicate gaps that have been introduced for optimal alignment of the sequences.

1.5 to 2 h. All further steps were carried out as previously described (van de Wiel et al., 1990). Fifty serial sections from four nodules of different ages were placed on one microscope slide. Sense and antisense RNA probes were transcribed with T3 and T7 RNA polymerase, respectively, from pBS.12–1 in the presence of [α-^{35}S]UTP (Amersham). Hybridization to tissue sections was carried out as described (van de Wiel et al., 1990). High-stringency washes with 50% formamide at 55°C and RNase A treatment were performed as described (Zeller and Rogers, 1989). Slides were dehydrated, dried, coated with Kodak NBT2 autoradiography emulsion, and exposed at 4°C for 1 to 4 weeks. The emulsion was developed in D19 developer and fixed in Rapid Fixer (Kodak). The sections were stained in 0.05% toluidine blue, and coverslips were affixed to the sections with Eukitt (Calibrated Instruments). Photographs were taken with Kodak Tungsten 160 color slide film on a Zeiss Axiophot microscope.

RESULTS

Molecular Characterization of Three Alfalfa Clones That Hybridize to PsENOD12

Using the PsENOD12 cDNA (Scheres at al., 1990) fragment as a probe, we identified five phage clones in the initial screen of 500,000 plaques of the alfalfa nodule cDNA library. One insert was subcloned into pUC119 to make pUC.E12–0 as well as into the *SmaI* site of pBSM13+ to generate pBS.E12–0 for further analysis and for rescreening the library. The clones were originally designated E12 based on their hybridization to the PsENOD12 probe.

Three additional clones were isolated upon rescreening and the inserts were subcloned into pUC119, generating a 509-bp insert (designated pUC.E12–1), a 414-bp insert (pUC.E12–2), and a 1459-bp insert (pUC.E12–3) (Fig. 1). For convenience, these clones will henceforth be referred to as E12–1, E12–2, and E12–3. E12–1 was also cloned into the *SmaI* site of pBSM13+, generating pBS.E12–1, which was used for the in situ hybridization experiments.

The cDNA sequence of E12–1 is 509 bp long and contains an open reading frame of 336 bp (Fig. 1). The encoded protein is 112 amino acids long and consists of a putative 26-amino acid signal peptide, based on the rules of Von Heijne (1983), and a Pro-rich region built of 14 repeats of the consensus motif pentamer PPIYK (Pro-Pro-Ile-Tyr-Lys). The encoded protein has a calculated molecular mass of 12.6 kD including the putative signal peptide, and of 10 kD without it. The E12–2 clone is truncated at the 5′ end and the derived peptide is one amino acid shorter at the carboxy terminus than the calculated E12–1 peptide (Fig. 1).

The 5′ region of E12–3 (nucleotides 1–794) does not contain an open reading frame for a peptide >36 amino acids (data not shown). E12–3 encodes a 111-amino acid peptide just 1 amino acid shorter at the carboxy terminus than the E12–1 peptide (Fig. 1). The molecular mass of the E12–3 peptide is calculated to be 12.5 kD if the putative signal peptide is included, and 9.8 kD if it is not. The translation start sites for the clones E12–1 and E12–3 best match the consensus translation start site TAAACAATGGCT for plants (Joshi, 1987). The other ATG triplets (underlined in Fig. 1) do not match the consensus as well, making it unlikely that they are utilized as translation start sites in vivo.

An analysis of the untranslated upstream region in E12–3 shows characteristic promoter elements, including an AGGA box (TGATTGAGACC) at position 733 and a TATA box (TATAAAA) at position 772 (Fig. 2). Partial homology to the consensus for the transcription start (CTCATCA; Joshi, 1987) was found 37 bp downstream of the TATA box at position 809 (Fig. 2). Moreover, the entire region 130 bp upstream of the translation start site is highly homologous (80%) to the PsENOD12B gene (Govers et al., 1991), with perfect conservation of the AGGA box, the TATA box, and the translation start consensus sequences (Fig. 2). Taken together, these observations indicate that the E12–3 sequence presented in Figure 1 most likely represents a genomic clone for the alfalfa cDNA clones.

Amino acid sequence comparison between the putative peptides encoded by the alfalfa clones and PsENOD12 revealed low homology between the Pro-rich regions (30% identity, 43% similarity), but a high degree of conservation between the putative signal peptides (92% identity, 100% similarity; Fig. 1). Homology of varying degree (<52%) was found to other Pro-rich proteins, including other nodulins. The highest degree of homology was found to a soybean cDNA coding for a repetitive Pro-rich protein (Averyhart-Fullhard et al., 1988) with 52% homology over a 110-amino acid region.

Temporal Expression Pattern

On northern blots, a probe made from the E12–1 clone hybridized to mRNA that is approximately 600 nucleotides long (Fig. 3). Cross-hybridization to larger RNAs of approximately 1500 and 2400 nucleotides was also occasionally observed (Fig. 4). Transcripts hybridizing to the E12–1 probe

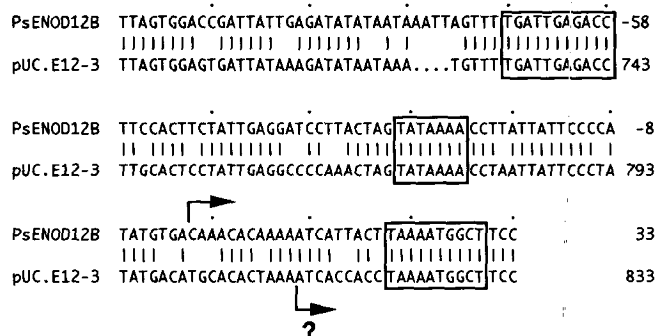

Figure 2. Sequence comparison of the promoter region of the PsENOD12B gene (Govers et al., 1991) with the highly homologous region of the pUC.E12–3 (E12–3) clone. The AGGA box (TGATT-GAGACC), TATA box (TATAAAA), and translation start site (TAAAATGGCT) are enclosed in boxes. The transcription start sites are indicated by arrows. The pea transcription start has been determined by S1 nuclease mapping (Scheres et al., 1990), whereas the transcription start for the alfalfa gene has not been determined experimentally. The coordinates for PsENOD12B are as in Govers et al. (1991), and those for E12–3 are as in Figure 1.

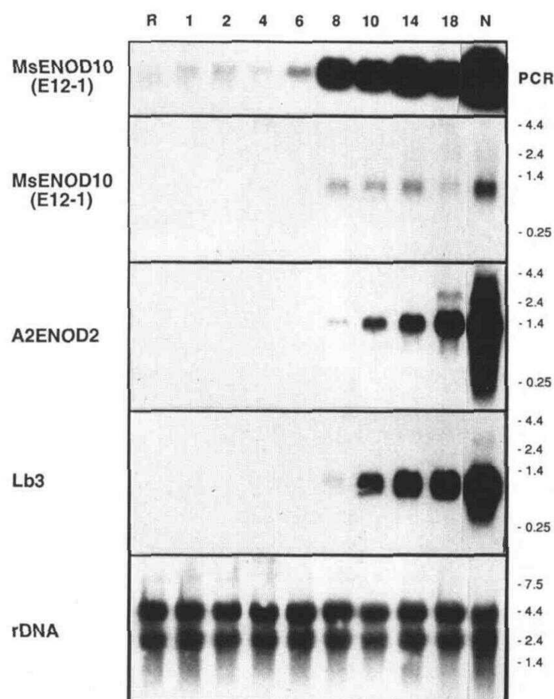

Figure 3. The PCR panel is a southern blot of the PCR products obtained after specific RNA amplification (see "Materials and Methods") and then probed with pUC.E12–1, one of the MsENOD10 clones. The remaining blots are northern blots of RNA isolated from alfalfa roots at specified times after inoculation with Rm1021. Numbers above the lanes indicate the days after inoculation when the roots were harvested. RNA from uninoculated roots (R) was isolated from 3-d-old plants, whereas nodule RNA (N) was extracted from Rm1021-induced nodules 21 d postinoculation. The blot was hybridized successively to probes derived from MsENOD10 (E12–1), A2ENOD2, Lb3, and rDNA. RNA size markers are given in kb.

were consistently detectable on northern blots of root RNA 8 d after inoculation with wild-type *R. meliloti* (Fig. 3, second panel). Nodules were first visible 6 d after inoculation under the growing conditions described (see "Materials and Methods"). We have detected mRNA that hybridized to the E12–1 probe in inoculated roots as early as 6 d postinoculation on some northern blots. In situ hybridization experiments on tissue sections of very young nodules confirmed this observation (Fig. 5B). The appearance of the E12–1-hybridizing transcripts parallels the onset of accumulation of MsENOD2 mRNA in inoculated root tissue (Fig. 3, third panel). E12–1-hybridizing transcripts were detected up to 43 d postinoculation with no obvious increase or decrease in transcript accumulation (data not shown).

Specific PCR amplification of mRNA using E12–1 primers and subsequent detection of PCR products on a Southern blot confirmed the occurrence of transcripts in alfalfa roots 6 d after inoculation. A faint hybridization signal to PCR products amplified from RNA isolated from roots 1 to 4 d after inoculation with *R. meliloti* was also seen (Fig. 3, first panel). However, this weak signal is probably due to background hybridization. In support of this supposition is the observation that a faint band was also detected in Figure 3, lane R (root), although this band was slightly smaller than those found in lanes 1, 2, 4, and 6.

Transcripts for the late nodulin leghemoglobin were barely detectable on northern blots 8 d after inoculation. However, a strong signal was observed 10 d after inoculation with *Rhizobium* (Fig. 3, fourth panel). We used an rDNA probe to show that the signals detected on the northern blot reflected changes in the concentration of the specific transcripts examined rather than fluctuations in RNA content from lane to lane. Figure 3, fifth panel, shows that the RNA levels for each lane were essentially equivalent.

Tissue Specificity

Next, we determined whether transcripts hybridizing to the E12–1 probe accumulated in plant tissues other than nodules or in nodules having altered development. Total RNA isolated from roots 10 and 20 d after inoculation with *R. meliloti* and from flowers, stems, leaves, and 22-d-old nodules was subjected to electrophoresis and prepared for northern analysis. Although RNA isolated from the inoculated roots and wild-type *R. meliloti*-induced nodules hybridized to the probe, transcripts were not detected in RNA isolated from aerial tissues (data not shown), which showed that mRNAs hybridizing to the E12–1 probe are specific to nodule tissue.

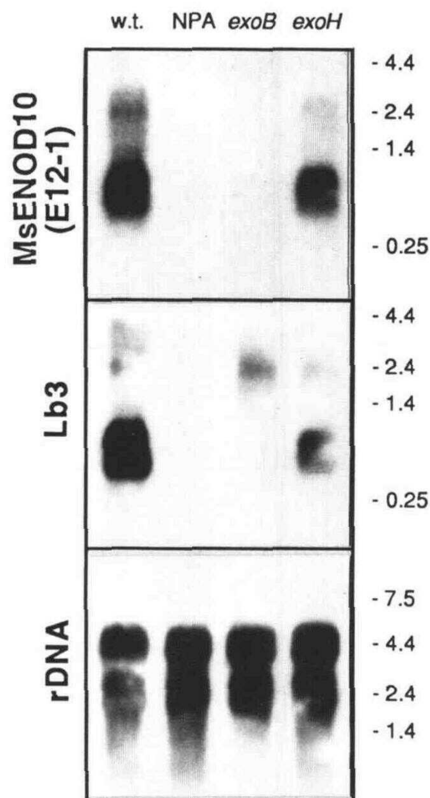

Figure 4. Northern blot of RNA isolated from nodules induced by Rm1021 (w.t.) or treatment with 10^{-5} M NPA (NPA), Rm5078 (*exoB*), and Rm7154 (*exoH*). The blot was successively probed with probes derived from MsENOD10 (E12–1), Lb3, and rDNA.

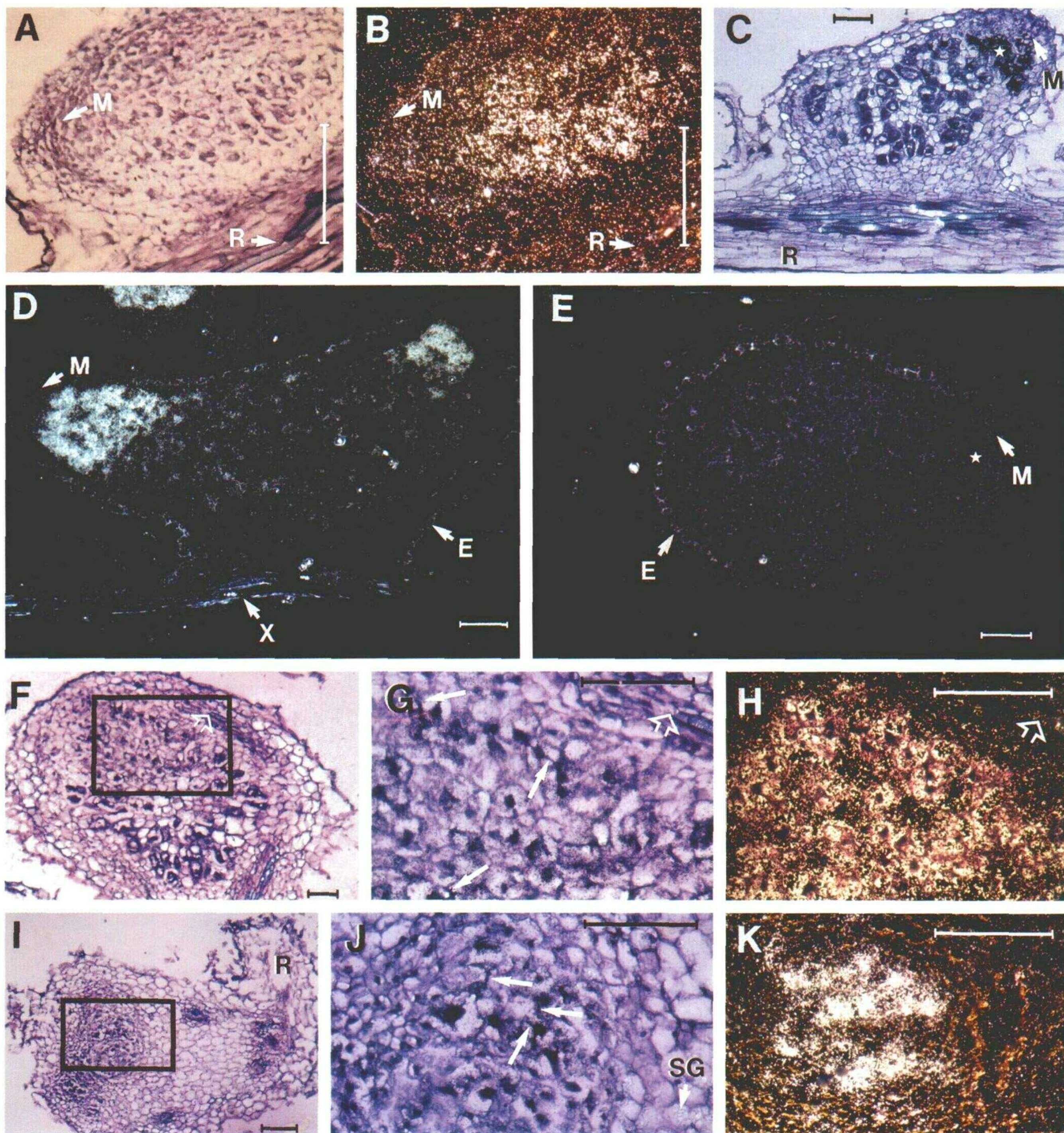

Figure 5. Localization of transcripts hybridizing to MsENOD10 by in situ hybridization. A, Bright-field illumination of a young nodule formed 6 d after spot-inoculation with Rm1021. The cells of the nodule meristem (M) are denser and more darkly staining than the interior cells of the nodule. The vascular system of the parent root is labeled (R). Magnification × 190. B, Dark-field photomicrograph of A. Silver grains corresponding to the positions of MsENOD10-hybidizing mRNAs are found in the interior of the young nodule. No transcripts are found in the meristem (M) or in the parent root (R). Magnification × 190. C, Bright-field photograph of a nodule 10 d after inoculation with Rm1021. Transcripts hybridizing to the antisense probe are indicated by the black silver grains deposited over the invasion zone (star), which is proximal to M. Magnification × 47. D, Dark-field illumination of a nodule 15 d postinoculation with Rm1021. The nodule is bifurcated; two meristems, one in median section (M), and two invasion zones are seen. Light-scattering silver-grain deposits indicating the presence of mRNAs hybridizing to the MsENOD10 antisense probe are present in the invasion zones. The xylem (X) and nodule endodermis (E) also scatter light. Magnification × 71. E, Nodule section hybridized to

RNA was isolated from nodules elicited by wild-type or mutant *R. meliloti* and also from pseudonodules formed in response to NPA treatment. The *R. meliloti exo* mutant-induced nodules are ineffective, free of bacteria, and lack a persistent nodule meristem (Yang et al., 1992). Infection threads abort in the peripheral cells of nodules induced by *R. meliloti exo* mutants except in the nodules induced by the *exoH* mutants (Leigh et al., 1987), where infection threads penetrate into the interior of the nodule in about 10 to 15% of the nodules formed. Pseudonodules formed in response to NPA treatment exhibit an even more atypical morphology. Although they have a nodule cortex and a central zone, they have a central vascular bundle rather than peripheral bundles, and no discrete nodule meristem. They are completely devoid of infection threads and bacteria (Hirsch et al., 1989).

We detected mRNAs hybridizing to the E12–1 probe only in wild-type *R. meliloti*-induced nodules and in *R. meliloti exoH*-induced alfalfa nodules. Transcripts were not present in nodules induced by the *R. meliloti exoB* mutant or in NPA-induced pseudonodules (Fig. 4). We also detected leghemoglobin transcripts in the nodules induced by wild-type and *exoH* mutant *R. meliloti* (Fig. 4).

Spatial Expression Patterns

To determine which cells within the nodule contain transcripts that hybridize to RNA made from pBS.E12–1, we performed in situ hybridization on alfalfa root nodule sections. Six-day-old alfalfa root nodules have a nodule meristem but few infected cells (Fig. 5A). Transcripts hybridizing to the antisense probe were detected in the interior of the developing nodule, but no transcripts were detected in other nodule tissues, including the nodule meristem (Fig. 5B). In a slightly older nodule, almost all of the cells of the central zone contained transcripts that hybridized to the antisense probe, in agreement with the results obtained for PsENOD12 by Scheres et al. (1990) (data not shown). In more mature nodules, transcripts hybridizing to the E12 antisense probe were restricted to the invasion zone of the nodule (Fig. 5, C and D). The bright signal associated with the vascular elements and also with the nodule and root endodermises in Figure 5D is probably due to suberized or lignified cell walls that scatter more light than surrounding tissues. Nodule sections that were hybridized to a sense probe also showed light scattering by endodermal cells (Fig. 5E). Silver grains were not detected in either root or nodule tissue following hybridization with the sense probe.

The absence of transcripts hybridizing to the antisense probe in root tissue (Fig. 3) was confirmed by the in situ hybridizations (Fig. 5, A-D).

Figure 5F shows an off-median section of a nodule comparable in age with that shown in Figure 5C. At higher magnification, both uninfected cells and cells containing infection threads (arrows) can be observed (Fig. 5G). Dark-field illumination shows that silver grains, indicating the presence of transcripts hybridizing to the antisense probe, were present in both uninfected and infected cells of the nodule invasion zone (Fig. 5H).

In contrast to wild-type *R. meliloti*-induced nodules, nodules induced by *exoH* mutants were mostly devoid of bacteria (Fig. 5I). Of the 20 nodules we examined using in situ hybridization, only one was infected with rhizobia. However, infection threads were found to have extended beyond the peripheral cells in approximately 20% of the nodules examined. Transcripts hybridizing to the antisense probe were detected in *R. meliloti exoH*-induced nodules into which infection threads had penetrated. Like wild-type *R. meliloti*-induced nodules, mRNAs hybridizing to the antisense probe appeared to be present in uninfected cells as well as in cells penetrated by infection threads (Fig. 5, J and K). However, [^{35}S]UTP probes do not provide fine enough resolution for us to localize the label exactly to one particular cell type.

Southern Analysis

Southern blot analysis of alfalfa DNA revealed that an E12–1 probe hybridized to several DNA fragments, suggesting that there may be a small gene family in alfalfa (Fig. 6A). We also found that E12–1 hybridized to genomic DNA isolated from the diploid *M. truncatula*. From the hybridization patterns obtained (Fig. 6A), we concluded that there are one or two genes hybridizing to the E12–1 probe in the *M. truncatula* genome.

Because of the sequence divergence in the Pro-rich regions of the alfalfa and pea clones, we probed the genomic Southern blots with the PsENOD12 cDNA fragment. Four fragments of *Eco*RI-digested *M. truncatula* DNA showed relatively strong hybridization to the PsENOD12 cDNA probe (Fig. 6B). In contrast, there was only very weak hybridization of genomic DNA isolated from alfalfa cv Iroquois to the PsENOD12 probe after an overnight exposure following a high-stringency wash (Fig. 6B). Only after longer exposure were weakly hybridizing bands visible on the film (data not shown). The bands that were detected after PsENOD12 hybridization and that did not also hybridize to the alfalfa probe are indicated by the asterisks (Fig. 6B).

(Continued from facing page.) an MsENOD10 sense probe. No silver grains are seen in the invasion zone (star) proximal to M. There is some light scattering by the suberized endodermis (E). Magnification × 71. F, A young nodule induced by Rm1021 and sectioned off-median so that the nodule meristem is not included in this micrograph. The boxed region corresponds to the invasion zone and is enlarged in G and H. Magnification × 47. G, High-magnification photograph of the nodule pictured in F. The arrows point to infection threads, whereas the open arrow points to a tracheary element that serves as a reference marker for G and H. Magnification × 190. H, Dark-field illumination of G. Silver grains are deposited over uninfected cells and cells penetrated by infected threads. Magnification × 190. I, A nodule induced by Rm7154. The boxed region is shown in higher magnification in J and K. Magnification × 47. J, High-magnification photograph of the nodule induced by Rm7154. The arrows point to infection threads. Starch grains (SG) are found in the uninfected nodule cells. Magnification × 190. K, Dark-field illumination of J. Silver grains are deposited over cells in the region where infection threads are found. Magnification × 190. The size bar in each photograph is 100 μm.

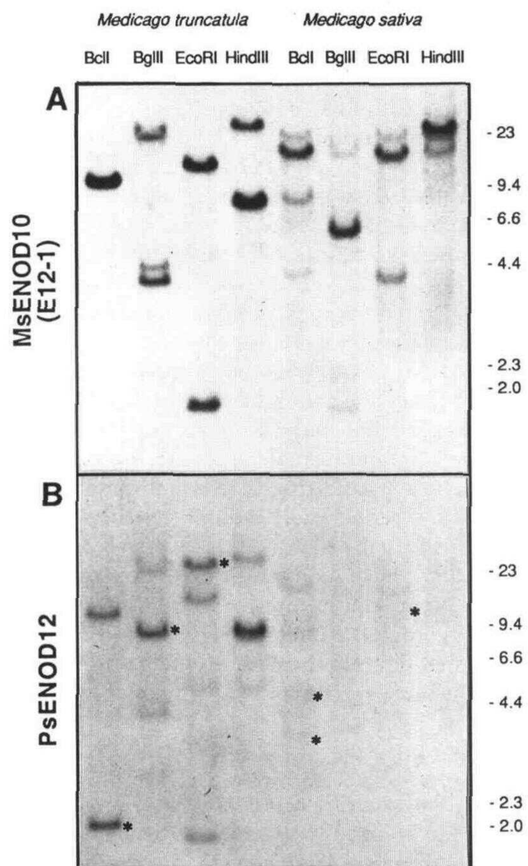

Figure 6. Southern blot of genomic DNA isolated from *M. truncatula* L. and *M. sativa* L. and digested with the restriction enzymes indicated above the lanes. Each lane contains approximately 2 μg of genomic DNA fragments. The blot was successively probed with MsENOD10 (E12–1) (A) and PsENOD12 (B). Each figure represents an overnight exposure. The bands detected only after PsENOD12 hybridization are marked with asterisks (*).

DISCUSSION

The goal of this study was to isolate an alfalfa cDNA clone homologous to the PsENOD12 clone and then to test whether the alfalfa ENOD12 homolog was expressed early during nodule development. Using a PsENOD12 cDNA fragment (Scheres et al., 1990) as a probe, we isolated three alfalfa cDNA clones, which were originally designated E12–1, E12–2, and E12–3 based on their hybridization to PsENOD12. Due to the similarity of the 5′ region of the E12–3 clone to the PsENOD12B gene promoter, we concluded that the E12–3 clone is probably a genomic clone. The isolation of a genomic clone from a cDNA library is not surprising because we have observed some residual DNA in our RNA preparations. PCR amplification of E12–1-hybridizing RNA from non-DNase-treated RNA samples isolated from alfalfa tissue always produced a DNA fragment that overlapped with the PCR products made from the RNA (data not shown). When the RNA samples were treated with DNase, the PCR product made from residual DNA was eliminated and only the RNAs gave rise to a specific PCR product (Fig. 3).

Recently, a gene was isolated from *M. truncatula*, the MtENOD12 gene, which has 83% nucleotide sequence identity in the signal peptide sequence and 76% nucleotide sequence identity in the Pro-rich repeat to the PsENOD12A gene (Pichon et al., 1992). These authors have proposed that the MtENOD12 and PsENOD12 genes have had a common evolutionary origin not only based on their striking DNA sequence similarities and near perfect alignment, but also on the similar temporal and spatial expression patterns that the two different genes exhibit. By contrast, the alfalfa clones we have isolated differ from the pea and *M. truncatula* ENOD12 clones in several ways. First, the alfalfa clones, although highly homologous to each other, are only weakly homologous to the pea and *M. truncatula* clones, except in their putative signal peptide. Second, the temporal expression pattern of the alfalfa clone differs from the timing of expression reported for PsENOD12 and MtENOD12. Both PsENOD12 and MtENOD12 are transiently expressed, with an increase in ENOD12 mRNAs within hours of *Rhizobium* inoculation followed by a decrease that occurs 14 d postinoculation. In contrast, transcripts hybridizing to E12–1 are not detected using northern and in situ hybridization analysis until 6 d postinoculation. In addition, we found, using northern blot analysis, that E12–1-hybridizing RNAs persist in mature nodules up to 43 d after inoculation (data not shown).

The PsENOD12 and MtENOD12 genes do not appear to require *Rhizobium*-mediated infection thread penetration for their expression. *Rhizobium* Nod factors have been shown to trigger ENOD12 gene expression in pea root hairs (Scheres et at., 1990), and NPA-induced pseudonodules of Afghanistan pea and NPA-treated pea root hairs have been shown to contain transcripts for PsENOD12 (Scheres et al., 1992), indicating that infection thread formation is not required for PsENOD12 gene expression. Similarly, MtENOD12 expression has been shown to be independent of infection thread formation (Pichon et al., 1992). Experiments in which *M. truncatula* roots are treated with NPA have not been done. In contrast, transcripts hybridizing to the alfalfa cDNA clone are not detected in NPA-induced pseudonodules or in *R. meliloti exoB* mutant-induced nodules. They are detected, however, in nodules that have been invaded by rhizobia such as the *R. meliloti exoH* mutant, which induces the development of infection threads that can extend into the interior tissues of the nodule. These results indicate that infection thread penetration may serve as the trigger for the expression of this alfalfa gene.

The evidence presented here indicates that the cDNAs we have isolated do not appear to be alfalfa homologs of PsENOD12, but rather correspond to a different alfalfa gene. Using Southern analysis, we determined that PsENOD12 hybridizes only weakly to the DNA isolated from alfalfa. However, Allison et al. (1993), by using PCR, recently isolated two *M. sativa* genes, designated MsENOD12A and MsENOD12B, that share sequence homology with the PsENOD12 genes; these are likely to be the PsENOD12 homologs. In *M. truncatula*, Southern analysis demonstrates that four *Eco*RI-digested DNA fragments hybridize to a PsENOD12-derived probe (Fig. 6B; D. Barker, personal com-

munication); one of these is MtENOD12 (Pichon et al., 1992). Thus, ENOD12 homologs exist in both *M. sativa* and *M. truncatula*.

The question remains as to the identity of the cDNA clones that we have isolated. Based on the evidence presented herein, we believe that these cDNAs represent mRNAs of a hitherto undescribed alfalfa gene, which we designate MsENOD10, to distinguish it from bona fide PsENOD12 homologs in alfalfa. This designation is based on the calculated molecular mass (10 kD) of the protein minus the signal peptide. A gene similar to MsENOD10 is present in *M. truncatula* (D. Barker, personal communication).

A number of unanswered questions remain about MsENOD10. For example, we do not know whether the MsENOD10 and MsENOD12 proteins serve the same function during nodulation. Transcripts are likely to be in the same place, although MsENOD12 mRNAs have not yet been definitively localized to the invasion zone of the nodule (A. Kondorosi, personal communication). We do not know whether the proteins, like their mRNAs, will also be found in the nodule invasion zone. The conservation of the signal peptide in both PsENOD12 and MsENOD10 is intriguing, but it might not serve any function other than targeting the proteins so that they are found in the same location in the nodule. Also, we do not know the identity of the *Rhizobium* signal that triggers MsENOD10 gene expression. On the basis of our results with the nodules formed in response to *R. meliloti exoB* (this report) and *exoF* mutants (Reddy et al., 1992), it would appear that *nod* gene products per se are not the stimulus for MsENOD10 gene expression. *R. meliloti exo* mutants have fully functional *nod* genes (Klein et al., 1988). Moreover, our preliminary experiments indicate that MsENOD10 transcripts are not detected in alfalfa seedling roots treated with culture filtrate containing *R. meliloti* Nod factor (Y. Fang and A.M. Hirsch, unpublished results). Other early nodulin genes, MsENOD12 and MsENOD40 (S. Asad, K. Wycoff, and A.M. Hirsch, unpublished data) are induced by Nod factor treatment (Y. Fang and A.M. Hirsch, unpublished results). These data suggest that the regulation of MsENOD10 gene expression is different from that of the other early nodulin genes, even though MsENOD10 is expressed at a relatively early stage of nodule development. We are currently testing this hypothesis.

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The GenBank accession numbers for the sequences of E12–1, E12–2, and E12–3 are M91074, M91075, and M91076, respectively.

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