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10. Blockage was determined by microscopic examination of each flea immediately after twice-weekly feedings on normal mice. Fleas that contained fresh blood only in the esophagus, anterior to the proventriculus and midgut, were defined as blocked (Fig. 1). The interval between infection and the appearance of blockage (the extrinsic incubation period) ranged from 5 to 51 days, with a median of 18 days. Male fleas tended to block sooner than females. Blocked fleas made prolonged, persistent attempts to feed, died within a week after development of the block, and contained more than 10^6 *Y. pestis* bacteria. These features are consistent with previous descriptions of the blockage phenomenon in *X. cheopis* (*1*, *11*).
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17. Adherence to proventricular cuticle was assayed by addition of 50 μl of Hank's balanced salt solution (HBSS) containing 10^6 bacteria and 1% bovine serum albumin to one to three freshly dissected *X. cheopis* proventriculi in the well of a 96-well tissue culture plate. We prepared each proventriculus by dissecting the midgut from a flea in sterile HBSS and bisecting it with a 27-g needle to expose the proventricular spines. The suspensions were incubated for 4 hours at room temperature. The proventriculi in each well were washed four times with HBSS and examined microscopically. Adherence was evaluated by enumeration of the bacteria associated with each proventricular preparation in two independent experiments with three replicates each for the *hms*$^+$ *Y. pestis* KIM6+ and the *hms*$^-$ KIM6 strains.
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Modification of Phytohormone Response by a Peptide Encoded by *ENOD40* of Legumes and a Nonlegume

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The gene *ENOD40* is expressed during early stages of legume nodule development. A homolog was isolated from tobacco, which, as does *ENOD40* from legumes, encodes an oligopeptide of about 10 amino acids. In tobacco protoplasts, these peptides change the response to auxin at concentrations as low as 10^{-12} to 10^{-16} M. The peptides encoded by *ENOD40* appear to act as plant growth regulators.

Legume nodule organogenesis is initiated by local dedifferentiation of root cortical cells activated by rhizobial Nod factors (*1*, *2*). Nod factors probably trigger cell divisions by inducing a local change of the auxin/cytokinin ratio (*3*). The expression of a few nodule-specific plant (nodulin) genes is induced by Nod factors (*4*, *5*). One of these genes, *ENOD40*, is first expressed in the root pericycle opposite to the nodule primordium (*4*, *6*–*8*). Expression precedes the induction of cortical cell divisions (*8*), which suggests that *ENOD40* may play a role in changing the response to phytohormones. To test this, we used tobacco as a model system.

To investigate *ENOD40* action, we transformed tobacco plants (*9*) with the construct 40-2/1-448, representing 448 base pairs (bp) of soybean *GmENOD40-2* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1) (*10*, *11*). Thirty percent of transgenic F_1 plants had one or two adventitious shoots at the base of the main shoot, as compared with none in untransformed tobacco plants (Fig. 2A). This suggests reduced apical dominance, raising the possibility that the transgenic plants were changed in terms of auxin me-

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tabolism or perception. One homozygous plant line, 11S-F2, containing a single copy of the *GmENOD40* transgene and displaying a similar phenotype, was used for further studies.

Checking the effect of *GmENOD40* expression at the cellular level, we found that at a 5.5 μM concentration of 1-naphtalene acetic acid (NAA), 11S-F3 protoplasts divided at a frequency similar to that of wild-type protoplasts (50 to 60%). However, at higher concentrations of auxin, at which the division frequencies of wild-type protoplasts declined (to 20 to 25%), 11S-F3 protoplasts displayed undiminished division frequencies (50 to 60%) (Fig. 3A). This tolerance of high auxin concentrations was also observed when wild-type protoplasts were transfected with *GmENOD40* linked to the CaMV 35S promoter.

The fact that *GmENOD40* is active in tobacco, a nonlegume, prompted us to isolate a tobacco homolog by polymerase chain reaction (PCR) (*10*). Primers representing two conserved regions of legume *ENOD40* (*4*, *6*, *7*, *12–14*) were used to obtain a tobacco *ENOD40* fragment from which a full-length cDNA was produced (Nt40/1-470) (Fig. 1) (*10*). A corresponding genomic sequence identical to the cDNA was also isolated. When transfected into tobacco protoplasts, 35S-*NtENOD40* conferred tolerance of high auxin concentrations in a manner similar to to that of 35S-*GmENOD40* (Fig. 1).

Sequence comparison of the tobacco and legume *ENOD40* clones revealed two conserved areas. The area at the 5′ end of all cDNAs (region 1) contained a highly conserved small open reading frame (ORF), starting with the first ATG available, encoding a peptide of 10 (tobacco), 12 (soybean), or 13 (pea, alfalfa, and vetch) amino acids (Fig. 3B). The second conserved sequence (region 2), located in the central part of *ENOD40*, lacked a conserved ORF.

We tested whether the small ORF of region 1 was actually translated in protoplasts by making a translational fusion between the ORF of *GmENOD40* and green fluorescent protein (GFP) (*15*) (Fig. 2E).

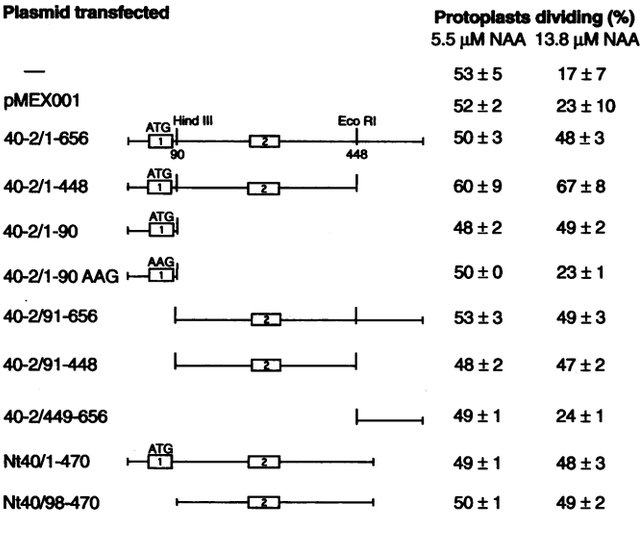

Fig. 1. Effect of transient expression of different *ENOD40* constructs on tobacco mesophyll protoplast cell division. *ENOD40* expression vectors 40-2/1-656 and Nt40/1-470 contain full-sized cDNA clones (*10*). The conserved regions 1 and 2 are denoted as white boxes. The Hind III and Eco RI restriction sites are indicated. Transient expression assays were performed as described (*19*). Wild-type (SR1) tobacco protoplasts were cultured in 0.9 μM kinetin and 5.5 or 13.8 μM NAA, respectively, and division frequencies were scored in three independent experiments. The percent of protoplasts dividing is given with the standard deviation.

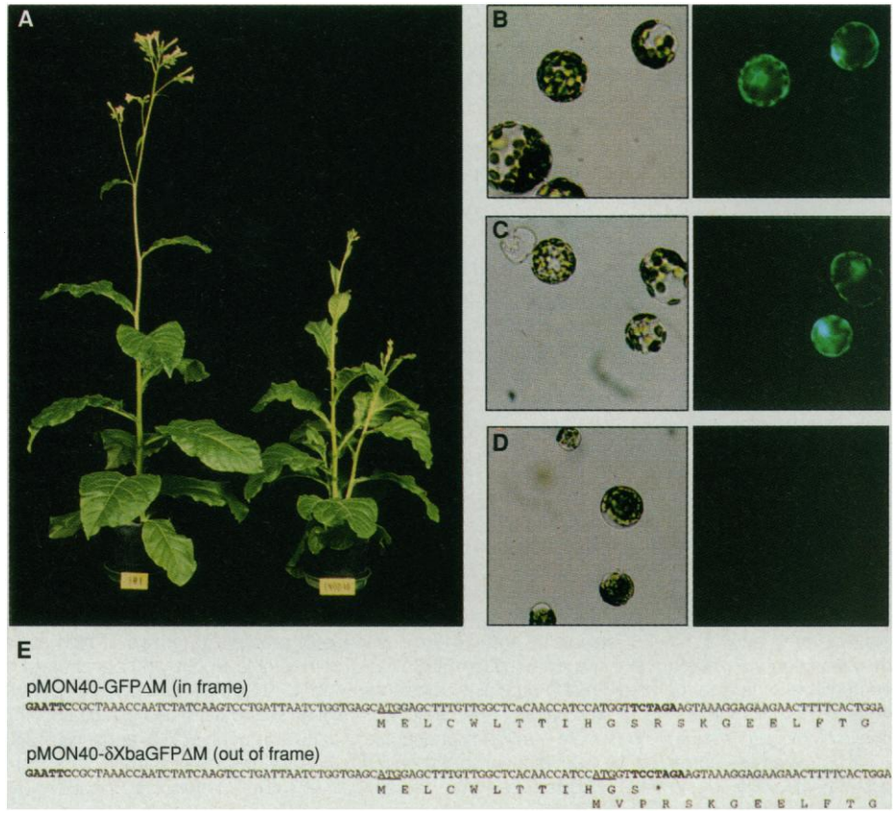

Fig. 2. (**A**) Phenotype of a heterozygous 35S-*GmENOD40* (40-2/1-448)–transformed tobacco plant (*20*). In contrast to the wild-type SR1 plant (left), the plant expressing *ENOD40* (4S-F1, right) has additional side shoots. (**B** through **D**) Detection of GmENOD40-2–GFP fusion products in tobacco mesophyll protoplasts. One day after transfection, expression of *GFP* was scored with a Leica DMBR microscope with the use of a Chromatechnology 41014 GFP filter. Photographs were taken under a bright field (left panels) and with a GFP filter (right panels). (B) Protoplasts transfected with pMON-GFP; (C) protoplasts transfected with pMON40-GFPΔM; and (D) protoplasts transfected with pMON40-δXbaGFPΔM. About 20% of protoplasts transfected with the in-frame pMON-40-GFPΔM construct showed fluorescence (C) and fluorescence was detectable in none of the protoplasts transfected with the out-of-frame construct (D). (**E**) Sequences of vectors pMON40-GFPΔM and pMON40-δXbaGFPΔM, representing in-frame and out-of-frame translational fusions of the peptide-encoding part of *GmENOD40-2* and *GFP* (*15*, *21*, *22*) used for transfection into tobacco protoplasts. (In the out-of-frame construct, a new ATG has been introduced into the *GFP* ORF). pMON-GFP was used as a positive control.

Protoplasts transfected with this construct showed similar amounts of GFP activity as was seen with a GFP construct containing its own translational start (*15*) (Fig. 2E). An out-of-frame *GmENOD40-GFP* construct with a new ATG in frame with the *GFP* ORF (Fig. 2E) was inactive. Thus, the *GmENOD40* ORF is indeed translated in protoplasts with its ATG and 5′ untranslated region functioning as a start site. This observation was extended by the finding that a subclone containing the ORF (40-2/1-90) induced tolerance of high auxin concentrations in transfected protoplasts, whereas a clone in which the ATG was replaced by AAG (40-2/1-90 AAG) did not (Fig. 1).

In vitro–synthesized GmENOD40-2 and NtENOD40 peptides conferred tolerance of high auxin concentrations in wild-type tobacco protoplasts when added exogenously (Fig. 3C). Half-maximal activity was reached at approximately 10^{-12} M concentration with the soybean peptide as compared with 10^{-16} M with the tobacco peptide. Control peptides were inactive (Fig. 3C).

The high frequency of transfected protoplast division at high auxin concentrations was suggestive of the release of a compound conferring tolerance to wild-type protoplasts. This compound is chymotrypsin-sensitive (*8*), and an enzyme-linked immunosorbent assay (ELISA) with an antibody against GmENOD40 peptide demonstrated the presence of GmENOD40 peptide in the medium of 11S-F3 but not of wild-type protoplasts. Furthermore, the GmENOD40 peptide is found in nodules but not in the roots of soybeans (Fig. 4). This demonstrates that the ENOD40 peptide is formed in tobacco protoplasts and root nodules, which is consistent with the occurrence of *ENOD40* RNA in monosomes of pea (*8*) and alfalfa root nodules (*13*). Because *ENOD40* is expressed in dividing cortical cells during legume nodule formation, and the induction of *ENOD40* expression in the pericycle precedes the mitotic activation of cortical cells, it is probable that the ENOD40 peptide plays a role in the start of nodule organogenesis (*4*, *6*–*8*). The accumulation of GmENOD40 in the medium tempts one to speculate that it is involved in cell-to-cell communication between pericycle and cortex. To answer the question of how ENOD40 alters the response of root cells to auxin, it will be essential to correlate the in situ expression pattern of *ENOD40* and its effect on the distribution of active auxin species in the different root cell types when cortical cells become mitotically activated.

Several groups have previously reported the absence of a long ORF in *ENOD40*. This, as well as computer analyses predicting that *ENOD40* RNA has a tendency to form secondary structures such as untranslated regions of mRNAs, led to the hypothesis that *ENOD40* is active as an RNA (*7*, *12*, *13*). Our finding that the *ENOD40* ORF is translated and the corresponding peptide is functional in causing tolerance of high auxin concentrations eliminates the basis for this hypothesis. Nevertheless, sequence comparison of different *ENOD40* clones shows the most conservation in region 2, which is located at the 3′ untranslated part of *ENOD40* mRNAs. This suggests that this part of the mRNAs might have a function. Constructs derived from *GmENOD40-2*, containing region 1 (40-2/1-90), region 2 (40-2/91-448 and 40-2/91-656), and the nonconserved 3′ end (40-2/449-656), and from the tobacco homolog containing re-

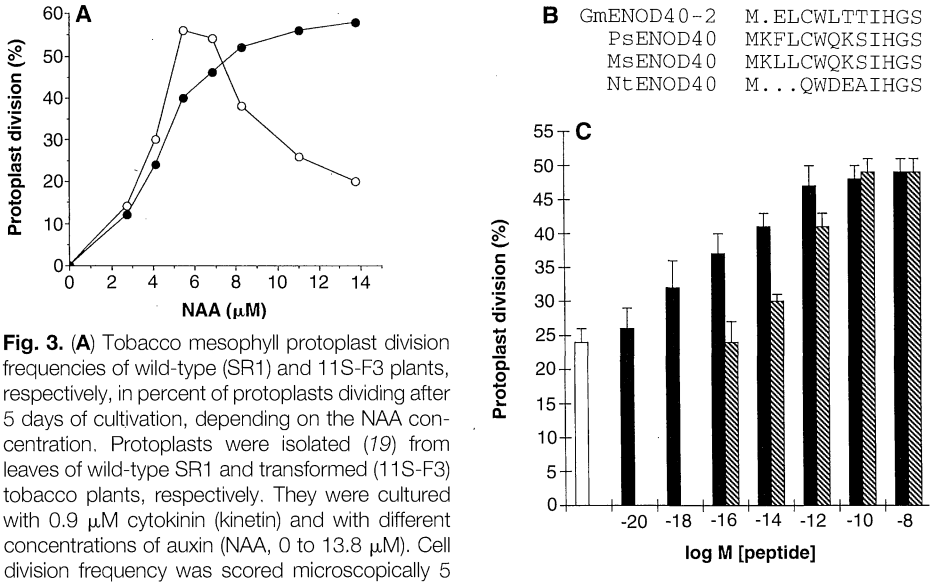

Fig. 3. (**A**) Tobacco mesophyll protoplast division frequencies of wild-type (SR1) and 11S-F3 plants, respectively, in percent of protoplasts dividing after 5 days of cultivation, depending on the NAA concentration. Protoplasts were isolated (*19*) from leaves of wild-type SR1 and transformed (11S-F3) tobacco plants, respectively. They were cultured with 0.9 μM cytokinin (kinetin) and with different concentrations of auxin (NAA, 0 to 13.8 μM). Cell division frequency was scored microscopically 5 days after isolation in three independent experiments. The variation between samples from different repeats was below 10%. The values from one representative experiment are given. (**B**) Sequence alignment of the ENOD40 encoded peptides from soybean (*4*), pea (*4*), alfalfa (*13*), and tobacco plants (*23*). A comparison of ENOD40 sequences from soybean, pea, alfalfa, and tobacco is available on the World Wide Web at http://gcg.tran.wau.nl/MolBio/ENOD40.html. (**C**) Division frequencies of tobacco mesophyll protoplasts in high auxin concentrations, in the presence of different concentrations of ENOD40 peptides. Filter-sterilized peptides (synthesized by Research Genetics without the NH_2-terminal methionine residues) were added to freshly isolated wild-type tobacco protoplasts. Division frequency was scored microscopically after 5 days of incubation in the presence of 13.8 μM NAA. Averages of three independent experiments are given. Black bars, tobacco peptide; striped bars, soybean peptide; white bar, background (H_2O). With control peptides MELMFATTARAT and RYLEYEAPTIPEDCGSLVIAHIGGK (*22*), only background amounts of cell division were found (49 ± 2% at 5.5 μM NAA and 22 ± 4% at 13.8 μM NAA).

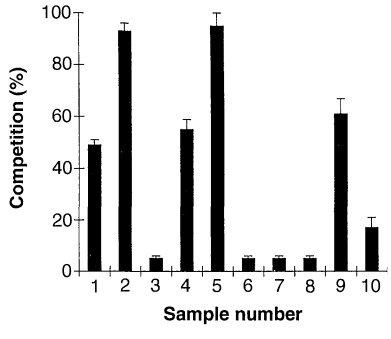

Fig. 4. Results of ELISA to detect GmENOD40-2 peptide. A mouse polyclonal antibody against GmENOD40-2 peptide coupled to keyhole limpet hemocyanin (KLH) was obtained (Eurogentec, Belgium). The specificity of this antibody for GmENOD40-2 peptide was determined in an ELISA using KLH, KLH coupled to GmENOD40-2 peptide, synthetic GmENOD40-2 (sample 1, 25 ng; sample 2, 100 ng), and NtENOD40 (sample 3) peptides in a competition assay for binding of the antibody to antigen (GmENOD40 peptide coupled to KLH)-coated microtiter plates. The antibody is specific for the soybean ENOD40 peptide (compare samples 1, 2, and 3). The presence of antigen in the medium of 40-2/1-448–transfected protoplasts (sample 4) (Fig. 1) and of 11S-F3 (sample 5) and wild-type (sample 6) protoplasts grown at high auxin concentration was determined in a competition ELISA. In a comparable ELISA, the absence of a competitive antigen in the medium of protoplasts transfected with 40-2/91-448 (sample 7) (Fig. 1) or 40-2/91-656 (sample 8) (Fig. 1) was proven. The rate of competition was determined by assay of the conversion of *p*-nitrophenyl phosphate by alkaline phosphatase at 405 nm in an ELISA reader (SLT 340 ATTC). Values are corrected for KLH binding to the antibody and are means of three independent experiments. Preimmune serum did not recognize the antigen (*20*). The GmENOD40 peptide was shown to be present in extracts of 15-day-old soybean nodules (sample 9) but not in roots (sample 10).

gion 2 (Nt40/98-470) were tested in protoplasts. Of these, only the *GmENOD40-2* construct lacking regions 1 and 2 did not stimulate cell divisions at 13.8 μM NAA (Fig. 1). So an RNA lacking region 1 but containing the untranslated region 2 causes a similar response in transfected protoplasts as does the ENOD40 peptide.

It is plausible that region 2 mediates its effect by stimulating the synthesis of the endogenous tobacco ENOD40 peptide. This idea is supported by the observation that the conditioned medium of protoplasts transfected with Gm40-2/91-448 contains a chymotrypsin-sensitive compound that confers tolerance of high auxin concentration to wild-type tobacco protoplasts (*8*). This compound was not recognized by the antiserum to GmENOD40, in contrast to the proteinaceous compound accumulating in the medium of Gm40-2/1-448–transfected protoplasts (Fig. 4). Because the tobacco ENOD40 peptide is not recognized by this antiserum (Fig. 4), it seems likely that this peptide accumulates in the medium upon transfection with the Gm40-2/91-448 construct. The molecular mechanism by which region 2 may be active remains to be solved. Thus, perhaps the 3′ untranslated region has the ability to stimulate expression of the endogenous *NtENOD40* gene, but a more probable explanation could be that region 2 is a translational regulating sequence. Studies on the *Drosophila* morphogen BICOID showed that it represses the translation of *caudal* mRNA by binding to a 3′ untranslated sequence (*16*). Thus, in protoplasts transfected with region 2, the RNA might titrate out a translational inhibitor that binds to the tobacco *ENOD40* mRNA.

Generally, biologically active peptides are synthesized as inactive precursors, whereas in the case of *ENOD40*, the primary translation product is the biologically active molecule. Until now, only prokaryotic genes have been identified that encode small active peptides, such as microcin C7, a heptapeptide inhibiting protein synthesis in enterobacteriaceae (*17*). In plants, one active peptide, systemin, has been identified. Systemin is formed as a precursor and is involved in the systemic induction of proteinase inhibitor genes (*18*). Our studies show that *ENOD40* encodes a peptide that modulates the action of auxin. Because of its proteinaceous nature and the low concentration at which it is active, ENOD40 could be considered as a plant growth regulator that alters phytohormone responses and occurs in legumes as well as nonlegumes.

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20. The 35S-*GmENOD40* construct 40-2/1-448 was used for *Agrobacterium tumefaciens* strain GV3101(pMP90RK)-mediated leaf disc transformation (*13*, *19*) of tobacco (*Nicotiana tabacum* cv. Havana SR1) [P. Maliga, A. Sz.-Breznovits, L. Marton, *Nature New Biol.* **244**, 29 (1973)]. Two plants expressing ENOD40, as shown by Northern (RNA) blot analysis (*8*), called 4S-F1 and 11S-F1, were self-pollinated. The progeny (11S-F3) of one homozygous plant of the F_2 of 11S (termed 11S-F2) was used for further analysis (in protoplast experiments).
21. To construct in-frame and out-of-frame GmENOD40-2–GFP translational fusions, a DNA fragment containing the 5′ leader and the peptide-encoding sequences of *GmENOD40-2* as well as the coding part of GFP [J. Haseloff and B. Amos, *Trends Genet.* **11**, 328 (1995)] were generated by PCR. The DNA fragments were cloned into pMON999 [H. van Bokhoven, J. Verver, J. Wellink, A. van Kammen, *J. Gen. Virol.* **74**, 2233 (1993)]. The nucleotide sequences of the inserts were determined, and two clones [pMON40-GFPΔM and pMON40-δXbaGFPΔM, representing in-frame and out-of-frame translational fusions of *GmENOD40-2* and *GFP* (Fig. 2E)] were used to transfect tobacco protoplasts (*14*). For a positive control, the *GFP* DNA fragment was cloned into pMON999.
22. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
23. H. Franssen *et al.*, data not shown.

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