Soybean *ENOD40* encodes two peptides that bind to sucrose synthase

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***ENOD40* is expressed at an early stage in root nodule organogenesis in legumes. Identification of *ENOD40* homologs in nonleguminous plants suggests that this gene may have a more general biological function. *In vitro* translation of soybean *ENOD40* mRNA in wheat germ extracts revealed that the conserved nucleotide sequence at the 5′ end (region I) encodes two peptides of 12 and 24 aa residues (peptides A and B). These peptides are synthesized *de novo* from very short, overlapping ORFs. Appropriate ORFs are present in all legume *ENOD40*s studied thus far. In this case small peptides are directly translated from polycistronic eukaryotic mRNA. The 24-aa peptide B was detected in nodules by Western blotting. Both peptides specifically bind to the same 93-kDa protein, which was affinity purified from soybean nodules. Using peptide mass fingerprinting, we identified this binding protein as nodulin 100, which is a subunit of sucrose synthase. Based on our data we suggest that ENOD40 peptides are involved in the control of sucrose use in nitrogen-fixing nodules.**

translation | short open reading frames | peptide signals

The nodule on the roots of legumes is an organ induced by rhizobia in which they are hosted intracellularly and where they find an ideal environment for symbiotic nitrogen fixation. The plant genes that are specifically induced by nodulation factor-secreting rhizobia during early stages of nodule development have been termed early nodulin (*ENOD*) genes. Among these genes, *ENOD40* is one of the earliest nodulins and appears to play an important role in root nodule organogenesis. *ENOD40* is induced by nodulation factors and the phytohormone cytokinin, and its expression precedes the first cortical cell division (1, 2). In mature nodules, the expression of *ENOD40* has been detected in cells surrounding vascular bundles (3, 4). In addition, this gene also is expressed at low levels in stem and root cells (3). Recent work (5) has revealed *ENOD40* homologs also in the monocotyledonous plants rice and maize. *ENOD40* is therefore widespread in the plant kingdom, suggesting that it may have a general biological function.

A remarkable feature of legume *ENOD40* genes is that they contain only short ORFs. Therefore, it was initially proposed that this gene functions as an RNA (6, 7). All *ENOD40* genes studied thus far contain two highly conserved regions. Recently, it was reported that the 5′ located conserved region I of soybean (*Glycine max*) *ENOD40* encodes a small peptide (8). This work claimed that the peptide renders tobacco cells insensitive to high concentrations of auxin. However, these data were obtained by counting tobacco cells undergoing division and could not be reproduced by using other proliferation assays (9). Moreover, a study with transgenic clover containing an auxin-responsive promoter-β-glucuronidase (*GUS*) fusion failed to show the involvement of this peptide in perturbing auxin balance (10). Although genetic approaches using translational fusions were used to study *ENOD40* expression (11, 12), no direct biochemical evidence has been presented showing that such short ORFs can be translated in eukaryotes (13).

Using translation in wheat germ extracts we show that two small peptides of 12 and 24 aa are directly synthesized from soybean *ENOD40* mRNA. We also report the affinity purification and identification of a protein from nodules that specifically binds both peptides.

Materials and Methods

Plant Materials. Soybean plants (*Glycine max* cv. Jutro) were grown in nitrogen-free medium in a growth chamber at 26°C under a photoperiod of 16 h. Inoculation of plants with *Bradyrhizobium japonicum* USDA 110 was performed directly upon sowing, and nodules were collected 4 wk after inoculation. Uninfected soybean plants were cultured in the same way. Nodules and uninfected roots were frozen in liquid nitrogen immediately after harvesting and stored at −70°C.

Construction of Plasmids. The 0.68-kb fragment of *Glycine max ENOD40* cDNA (European Molecular Biology Laboratory database, accession no. X69154; ref. 4) was cloned into the *Bam*HI and *Sal*I sites of pBluescript SK (Stratagene). Single point mutations in the ATG of ORF A or ORF B were introduced into the *ENOD40* gene by using the QuikChange site-directed mutagenesis kit (Stratagene). The mutations were confirmed by DNA sequencing.

Synthetic Peptides. Peptides were synthesized by Neosystem (Strasbourg, France) according to the amino acid sequence deduced from the nucleotide sequence of ORF A (MELCWLTTIHGS) and ORF B (MVLEEAWRERGVRGEGAHSSHSLT) of soybean *ENOD40* cDNA. Synthetic analogs were MELSWLTTIHGS and MELCWLTTIGGG for peptide A and EVGHSRAWHASEGLRMTSRLEGVE and MVLQQAWGQGGVGGQGAYSSYSLT for peptide B. Peptides were labeled by coupling an additional biotinylated lysine residue to the C terminus of the sequence. Control peptides were from Bachem and biocytin (biotinyl-L-lysin) was from Sigma. Control A was a biotinylated analog of the cGMP-dependent protein kinase substrate with the sequence RKISASEFDRPLR. Control B was a biotinylated fragment (residues 44–68) of human PTH (RDAGSQRPRKKEDNVLVESHEKSLG). Purity of each peptide was assessed by HPLC.

***In Vitro* Translation Assays.** Plasmid DNA, with *ENOD40* cDNA cloned downstream of the T3 RNA polymerase promoter, was linearized with *Kpn*I and transcribed into capped mRNA by using the T3 Cap-Scribe system (Roche Diagnostics). Capped mRNA was translated in wheat germ extract in the presence of [^{35}S]methionine (30 μCi, Amersham Pharmacia). The *in vitro* translation reaction contained 0.5 μg of RNA, 7.5 μl of wheat germ extract (Promega), 60 mM potassium acetate, and a mixture of protease inhibitors (Complete, EDTA-free and bestatin, Roche Diagnostics) in a final volume of 25 μl and was incubated at 25°C for 60 min. Radiolabeled *in vitro* translation

Abbreviations: ENOD, early nodulin; SCX, strong-cation exchange; SuSy, sucrose synthase; TFA, trifluoroacetic acid.

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products were isolated by RP C18 solid-phase extraction. The reaction mixture was acidified by adding 175 μl of 0.1% trifluoroacetic acid (TFA) and then applied to a solid-phase extraction column (Vydac, Hesperia, CA, 218TPB13), previously equilibrated with 0.1% TFA in water. The column bed was first washed with 0.1% TFA/H_2O and subsequently with 0.1% TFA/10% acetonitrile. Retained peptides were eluted with 0.1% TFA in acetonitrile and evaporated to dryness.

HPLC of Peptides. Solid-phase extracted peptides were first purified by ion-exchange HPLC. Samples were dissolved in solvent A (5 mM phosphate, pH 3.0/25% acetonitrile) and applied to a polysulfoethyl aspartamide column (200 × 4.6 mm, 5-μm particle size, The Nest Group, Southborough, MA). Strong-cation exchange (SCX) HPLC was carried out with a 60-min linear gradient from 0 to 100% B, at 0.7 ml/min, where B is solvent A containing 0.5 M NaCl. Radiolabeled compounds were detected by on-line radioactivity monitoring (Berthold, Wildbad, Germany). The radioactive fraction was collected and applied directly to a RP-HPLC column (Zorbax 300SB-C18 column, 250 × 4.6 mm, 5 μm). The column, operated at 0.8 ml/min, was eluted with a 60-min linear gradient from 0 to 100% B, where solvent A is 0.1% TFA/water, and solvent B is 0.1% TFA/75% acetonitrile.

Preparation of Antipeptide Abs and Immunoprecipitation. Abs directed against peptides were produced by immunizing rabbits with synthetic peptides coupled to keyhole limpet hemocyanin. Peptide A (deduced from ORF A) was coupled to keyhole limpet hemocyanin through the glutamic acid whereas peptide B (deduced from ORF B) was coupled to the carrier protein through an additional tyrosine added to the C terminus of the sequence. Rabbit antisera against peptide-keyhole limpet hemocyanin were obtained from Neosystem. IgG fractions were prepared by purification on protein A-Sepharose CL-4B (Amersham Pharmacia). For the isolation of antigen-specific Abs, an affinity matrix was prepared by coupling either biotinylated peptide A or peptide B to streptavidin-agarose beads. Purification of polyclonal Abs on an antigen column was carried out as described (14). For the immunoprecipitation of ^{35}S-labeled peptides from extracts, we used the immunoprecipitation kit with protein A-agarose (Roche Diagnostics). Radiolabeled *in vitro* translation products were dissolved in tricine sample buffer (Bio-Rad), and the samples were boiled for 5 min. Peptides were analyzed by Tricine-SDS/16.5% PAGE (15) and autoradiography.

Immunodetection of Endogenous Peptides. Frozen nodule or root tissue (1 g) was ground in liquid nitrogen. The powder was thawed in 3 ml of extraction buffer containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM EDTA, 250 mM sucrose, and protease inhibitors (Complete and bestatin). The suspension was then homogenized at 4°C with an Ultra-Turrax T8 (IKA, Staufen, Germany) and centrifuged for 5 min at 15,000 × *g*. To the resulting pellet, 200 mM Tris·HCl (pH 6.8), 40% glycerol, 4% SDS, and 5% 2-mercaptoethanol were added. Samples were boiled for 10 min and colorless tricine-SDS/PAGE was performed in 16.5% gels (16). Peptides were electroblotted to a Sequi-Blot poly(vinylidene difluoride) membrane (Bio-Rad) by using Towbin buffer with 30% methanol (17), and the membrane was blocked with 5% BSA for 30 min. The immobilized antigens were detected with affinity-purified antipeptide Abs followed by incubation with alkaline phosphatase-conjugated second Abs by using the ProtoBlot II AP system (Promega). Gels were calibrated with prestained molecular weight markers (MultiMark, Invitrogen).

Affinity Purification of Peptide-Binding Proteins. Soybean nodules were ground in liquid nitrogen and homogenized in 10 mM sodium phosphate buffer (pH 7.6) containing 500 mM NaCl and protease inhibitors at 4°C. After centrifugation at 8,000 × *g* for 20 min, the supernatant was passed through a PD-10 desalting column (Amersham Pharmacia). The fraction excluded from the gel matrix was collected, and a detergent mix was added to achieve a final concentration of 0.1% Nonidet P-40 and 0.05% sodium deoxycholate in the extract. To reduce nonspecific binding, extracts (1 ml) were mixed with 100 μl of a 50% suspension of washed streptavidin-agarose beads (Pierce) and tumbled for 45 min at room temperature. Biotinylated peptides (20 μg) were incubated with pretreated supernatant for 1 h at room temperature followed by an incubation with 100 μl of streptavidin-agarose beads for a further 1 h. The beads were washed six times (10 min each) with 1.0-ml portions of buffer (10 mM sodium phosphate, pH 7.6/500 mM NaCl/0.1% Nonidet P-40/0.05% deoxycholate). Bound proteins were released by boiling for 5 min in SDS/PAGE sample buffer. Samples were separated on 10% SDS/polyacrylamide gels, and proteins were stained with SYPRO ruby (Molecular Probes) or colloidal Coomassie (NOVEX, San Diego).

Peptide Mass Fingerprints. Protein bands were excised from colloidal Coomassie blue-stained gels and digested in-gel with trypsin (18). The peptide solution was desalted on a microcartridge (18) and analyzed on a matrix-assisted laser desorption ionization-time-of-flight mass spectrometer (Reflex III, Bruker) equipped with a Scout 384 ion source. Peptide mass spectra were recorded and the National Center for Biotechnology Information protein database restricted to eukaryotes was screened with the search engine PROFOUND (19).

Peptide-Binding Assays. Peptide-binding assays were performed with partially purified sucrose synthase (SuSy). Soybean nodules were homogenized with extraction buffer containing 5 mM DTT as described above. After centrifugation at 30,000 × *g* at 4°C, the supernatant (500 μl) was fractionated on a calibrated Superdex 200 HR 10/30 column (Amersham Pharmacia). The column was eluted with 20 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, and 200 mM NaCl (for binding assays with peptide A) or with 20 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, and 500 mM NaCl (for assays with peptide B) at a flow rate of 0.8 ml/min. The SuSy-containing fraction (≈1.6 ml) was collected, and protease inhibitors and a detergent mix were added to achieve a final concentration of 0.1% Nonidet P-40 and 0.05% deoxycholate. Binding reactions with 5 μg of biotinylated peptides and subsequent coupling to streptavidin-agarose beads were performed as described above. The beads were washed five times with 20 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 0.1% Nonidet P-40, and 0.05% deoxycholate containing 0.5 M NaCl (for peptide A-affinity matrix) or with the same buffer containing 1 M NaCl (for peptide B-affinity matrix). Before SDS/10% PAGE, the beads were washed once with 10 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, and 50 mM NaCl.

Preparation of Anti-SuSy Abs. SuSy was purified from soybean nodule extracts as described (20) and used to raise Abs in rabbits. Antiserum against SuSy was obtained from Eurogentec (Brussels), and IgG fractions were prepared by purification on Protein A-Sepharose.

Results

Identification and Characterization of *in Vitro* Translation Products from *GmENOD40* mRNA. Comparison of the nucleotide sequences of legume and nonlegume *ENOD40* revealed two highly conserved regions (regions I and II, ref. 5). An intriguing feature of *ENOD40* cDNAs is that they possess only short ORFs (Fig. 1*A*). To find out whether the predicted translation products encoded by soybean *ENOD40* are made, the corresponding cDNA was

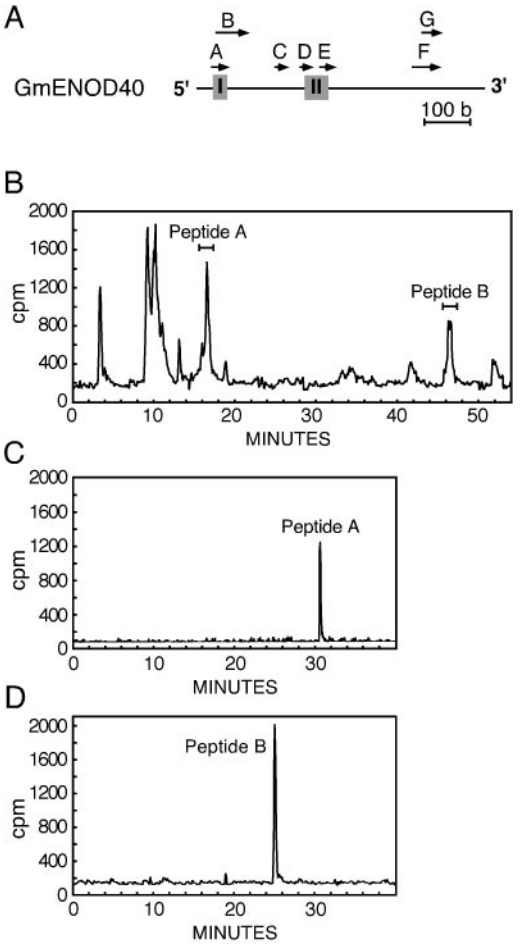

Fig. 1. HPLC analyses of the *in vitro* translation products synthesized from *GmENOD40* mRNA. (*A*) Location of possible ORFs and conserved regions in *GmENOD40* mRNA. Short ORFs are indicated by arrows. The conserved regions I and II are boxed. (*B*) Strong cation-exchange chromatography of the extracted *in vitro* translation products on a polysulfoethyl aspartamide column. SCX chromatography was carried out with a 60-min linear salt gradient at a flow rate of 0.7 ml/min. Radiolabeled peptides A and B were collected as indicated. (*C* and *D*) Zorbax C-18 RP-HPLC of the radioactive fractions indicated in *B*. The column was eluted with a linear acetonitrile gradient at a flow rate of 0.8 ml/min. The chromatograms in *B*, *C*, and *D* show radioactivity detected by an on-line radioactivity monitor.

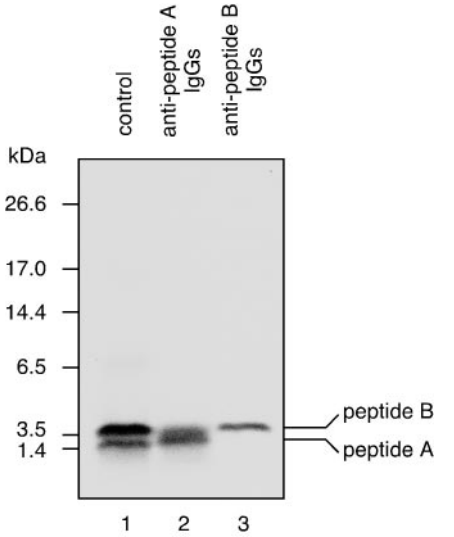

Fig. 2. Immunoprecipitation of *in vitro* translation products. [^{35}S]-Methionine-labeled peptides translated in wheat germ extracts from wild-type *GmENOD40* mRNA were solid-phase extracted (lane 1, control), immunoprecipitated with antigen-specific IgGs (lanes 2 and 3), and analyzed by Tricine-SDS/16.5% PAGE and autoradiography. For immunoprecipitation, affinity-purified Abs directed against ORF A- and ORF B-deduced synthetic peptides were used.

cloned downstream of the T3 RNA polymerase promoter and transcribed *in vitro*. Capped mRNA was subsequently translated in wheat germ extracts in the presence of [^{35}S]methionine. The peptidic translation products were separated from the bulk of unincorporated radiolabel by RP solid-phase extraction. Purification of extracted peptides was then achieved by using a strong cation-exchange HPLC column, which was particularly useful because the position of peptides eluting from this column increases monotonically according to their net positive charge at pH 3 (21). SCX HPLC analysis showed two radiolabeled translation products from *GmENOD40* mRNA, named peptide A and B, which elute at positions corresponding to peptides containing approximately two or six positively charged residues (Fig. 1*B*). These compounds coeluted with synthetic peptide A and peptide B, which were deduced from the nucleotide sequence of ORF A and ORF B, respectively (Fig. 1*A*). The radioactive fractions containing peptide A and B (Fig. 1*B*) were collected separately and applied directly to a C18 RP-HPLC column. This complementary separation technique separates on the basis of hydrophobicity. One peak coeluting with the corresponding synthetic peptide was obtained for each fraction (Fig. 1 *C* and *D*).

Immunoprecipitation of Translation Products. We raised polyclonal Abs against chemically synthesized peptides A and B coupled to keyhole limpet hemocyanin. The antipeptide Abs were immunoaffinity-purified on an antigen column and used to immunoprecipitate radiolabeled translation products of the wild-type *GmENOD40* mRNA. Tricine-SDS/PAGE followed by autoradiography demonstrated that peptide A (1.4 kDa) and peptide B (2.7 kDa) could be immunoprecipitated with the corresponding Abs (Fig. 2, lanes 2 and 3). Apart from these two peptides, no other translation products of the *GmENOD40* mRNA could be detected (Fig. 2, lane 1).

These antipeptide Abs were included in all subsequent *in vitro* translation reactions to protect synthesized peptides from proteolytic degradation (compare the translation of wild-type mRNA in Fig. 3*B* with Fig. 1*B*).

***In Vitro* Translation of Initiation Codon Mutants.** The results obtained from HPLC analyses of the *in vitro* translation products indicate that ORF A and B are the coding regions of peptides A and B. To provide further evidence that such short and overlapping ORFs are indeed translated, we introduced a point mutation in the AUG of ORF B (mutant M1) or in the AUG of ORF A (mutant M2) by using site-directed mutagenesis (Fig. 3*A*). The base exchange of M1 should abolish the synthesis of peptide B but would not affect the potential translation of ORF A. *In vitro* translation of the corresponding mutant mRNA M1 and subsequent SCX chromatographic analysis verified that translation of ORF B was blocked (Fig. 3*B*). A single base substitution in the initiation codon of ORF A (M2) resulted in expression of peptide B only (Fig. 3*B*).

***ENOD40s* from Various Legumes Encode Similar Peptides.** Reading frames corresponding to ORF A and ORF B are present in all *ENOD40* cDNAs sequenced until now. Recently, the amino acid sequence of soybean peptide A was compared to the predicted

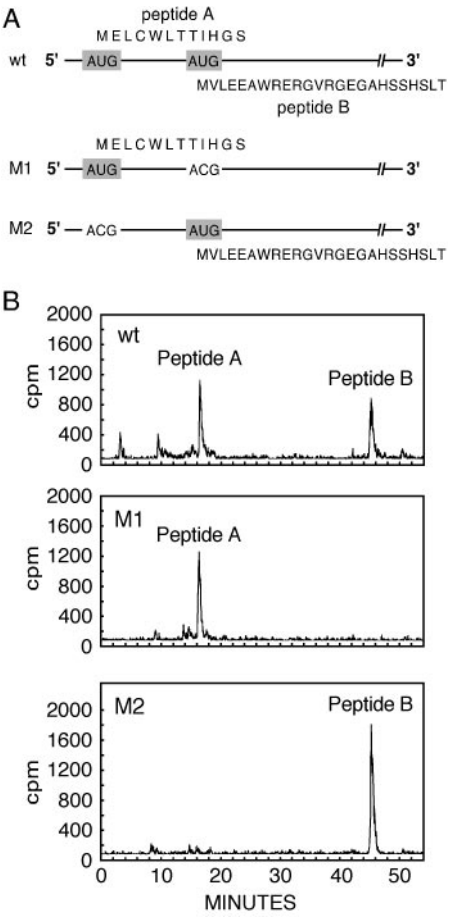

Fig. 3. Translation of mutant mRNAs in wheat germ cell-free system in the presence of anti-peptide Abs. (*A*) Schematic presentation of two small overlapping ORFs of region I located at the 5′ end of wild-type (wt) *GmENOD40* mRNA (ORF A and ORF B, Fig. 1*A*). Mutations in the initiation codons of ORF B and ORF A are indicated (mutant mRNAs M1 and M2). (*B*) *In vitro* translation of wt and mutant mRNAs in the presence of antipeptide A and antipeptide B Abs (50 μg each) and SCX chromatography of radiolabeled products.

sequence of other legumes (5), indicating that this small peptide is highly conserved. Alignment of the *ENOD40*-encoded peptide B from soybean to sequences in other legumes shows that all deduced peptides are highly charged (data not shown). The amino-terminal three amino acids MVL are fully conserved and are followed by at least one basic or acidic residue.

Detection of Peptides in Nodules. Using Western blot techniques, optimized for the analysis of small proteins and peptides, we examined whether the *ENOD40* translation products are present in mature soybean nodules. Fig. 4 shows that monospecific antipeptide Abs recognized peptide B in the insoluble fraction of nodule extracts but not in the same fraction obtained from roots. A band corresponding to peptide A could not be detected by Western analysis of nodule extracts by using monospecific antipeptide A Abs. Nevertheless, these Abs recognized several compounds with molecular masses >17 kDa on the poly(vinylidene difluoride) membrane (data not shown).

Isolation and Identification of a 93-kDa Peptide-Binding Protein. To isolate proteins that specifically bind to the peptides, we prepared extracts from mature soybean nodules and used these for affinity purification. Biotinylated peptides A or B were incubated for 1 h with the nodule extracts and were then bound to

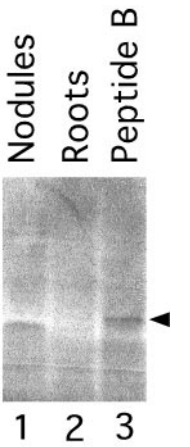

Fig. 4. Detection of peptide B in nodules. Samples were analyzed by tricine-SDS/16.5% PAGE, transferred to a poly(vinylidene difluoride) membrane, and detected with peptide B-specific Abs. Arrowhead marks the position of peptide B.

streptavidin-agarose beads. After extensive washings, the retained proteins were eluted and subsequently analyzed by SDS/PAGE. With both biotinylated peptides, this purification method yielded one major protein band of ≈93 kDa (Fig. 5, lanes 1 and 3). No protein was obtained by this procedure if biotinylated control peptides of similar size were used (Fig. 5, lanes 2 and 4).

These data suggest that both peptide A and peptide B recognize the same target protein. To test this, protein bands were excised from colloidal Coomassie blue-stained gels and subjected to in-gel tryptic digestion, and the resulting peptides were identified by matrix-assisted laser desorption ionization-time-of-flight spectrometry. Peptide mass fingerprints of peptide A- and peptide B-binding proteins were identical (data not shown). In addition, using these mass spectrometric peptide mapping data, 319,204 protein sequences from all known data

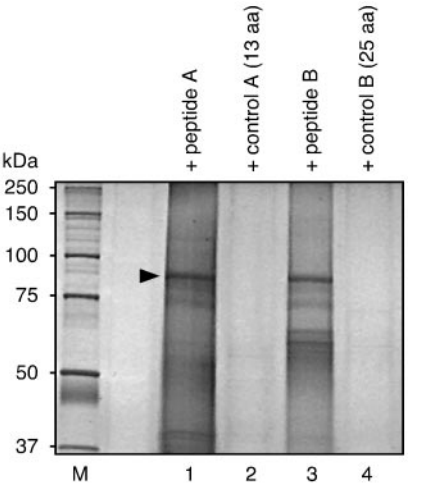

Fig. 5. Peptide A and peptide B bind to the same target protein in nodule extracts. Analysis by SDS/PAGE. Biotinylated peptides A and B (lanes 1 and 3) and the corresponding biotinylated control peptides (lanes 2 and 4) were incubated with extracts from soybean nodules and subsequently bound to streptavidin-agarose beads. Proteins isolated with the peptide affinity matrix were eluted, analyzed by SDS/10% PAGE, and stained with SYPRO ruby. The arrowhead marks the position of the 93-kDa peptide-binding protein. Sizes of molecular mass markers (lane M) are indicated.

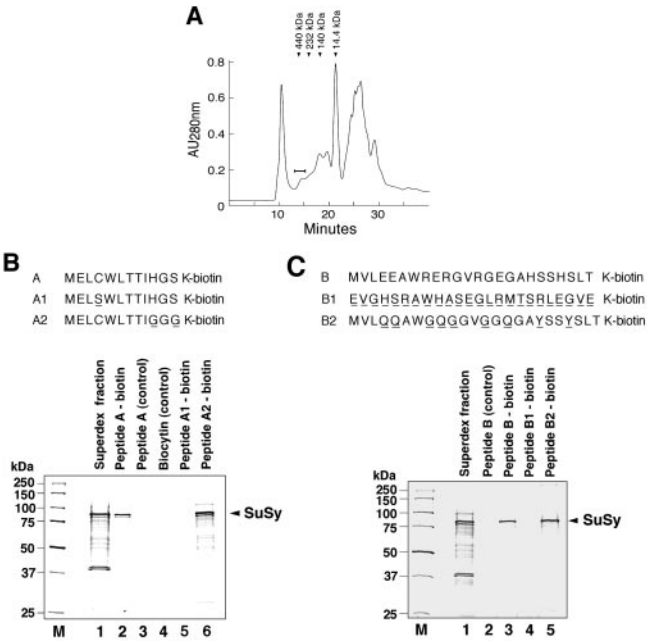

Fig. 6. Binding of wild-type and mutant GmENOD40 peptides A and B to SuSy. (*A*) SuSy was partially purified from soybean nodule extract by gel chromatography on Superdex 200. The SuSy-containing fraction used for binding reactions (bar) and the elution position of marker proteins ferritin, catalase, lactate dehydrogenase, and lysozyme are indicated (arrowheads). (*B* and *C*) Binding of biotinylated peptides and mutant derivatives to SuSy. Substitutions of amino acid residues in the corresponding peptides are underlined. SuSy was isolated from the Superdex fraction (lane 1) with peptide-streptavidin-agarose beads. Proteins bound to the affinity matrix were eluted by boiling in sample buffer, resolved by SDS/10% PAGE, and stained with SYPRO ruby. Lane M, molecular size markers. The authenticity of the SuSy protein band was verified by Western blotting with anti-SuSy Abs (data not shown).

sources (National Center for Biotechnology Information database) were screened with the search engine PROFOUND (19). The program consistently identified the peptide A- and peptide B-binding protein as soybean nodulin 100, which is a subunit of SuSy with an excellent score value of 2.39. The average deviation of the measured peptide masses compared to the theoretical values of soybean SuSy peptides (GI 3915872) was 0.026 Da with a sequence coverage of 53%.

By using immobilized peptides as an affinity matrix, we also could demonstrate that the level of SuSy protein is highly elevated in nodules compared to root tissue (data not shown), which is consistent with previous findings (20).

ENOD40 Peptides Specifically Bind to Purified SuSy. To study the structural requirements for peptide binding to SuSy, it was necessary to reduce proteolytic activity in the binding reaction. Therefore, we partially purified SuSy by gel chromatography on Superdex 200. SuSy eluted from the column at a position consistent with the calculated molecular mass of the homotetramer (371 kDa; Fig. 6*A*). We used this partially purified SuSy fraction in peptide-binding assays and found that biotinylated peptides A and B specifically bind to SuSy (Fig. 6 *B* and *C*).

Peptide A contains a Cys residue that is highly conserved in legumes, and conversion of this residue to Ser (peptide A1) strongly affected peptide A binding activity. This suggests that the sulfhydryl group of the Cys residue is essential for the interaction with SuSy and may be involved in disulfide bonding. In contrast, the conserved sequence His-Gly-Ser at the COOH-terminal portion of peptide A seems to be of minor importance because mutation to three consecutive glycines (peptide A2) did not affect binding to SuSy (Fig. 6*B*).

A striking feature of peptide B is that it contains many charged amino acid residues. To evaluate the functional importance of these residues in peptide B binding, we substituted all Glu, Arg, and His residues with Gln, Gly, and Tyr, respectively (peptide B2). In the binding assay, this mutated peptide B still interacted with SuSy, which indicates that the charged residues are not essential for binding (Fig. 6*C*). On the other hand, binding affinity was strongly reduced when a peptide analog was used in which the amino acid composition is identical to peptide B but the residues are arranged in a completely randomized sequence (peptide B1; Fig. 6*C*).

Discussion

Systemin was the first peptide signal discovered in plants (22). This systemically acting wound signal is rapidly transported through the plant and activates defense gene expression in tissues remote from the primary wound site. Recently, phytosulfokines have been identified, which are sulfated tetra- and pentapeptides with mitogenic activity in plant cell cultures (23). These findings, together with the identification of genes that encode putative peptide receptors showing similarity to animal receptors (24–26), indicate that peptides are important signal molecules in plants analogous to peptide hormones in animals and yeast. Plant defense signal systemin (27), the mitogenic phytosulfokines (28), and all animal peptide signals are released from larger precursors by proteolytic cleavage. We present here direct evidence for the *de novo* synthesis of two different peptides in a eukaryotic cell-free system. Both peptides are translated in wheat germ extracts from very short overlapping ORFs located at the 5′ end (conserved region I) of soybean *ENOD40* mRNA. Such an organization is unusual for eukaryotic mRNA and normally found among plant viruses (29).

Our conclusion that these small ORFs are indeed translated in plants is based on several lines of evidence. First, two radiolabeled *in vitro* translation products of 12- (peptide A) and 24-aa residues (peptide B) were obtained that coeluted from two different HPLC columns with synthetic peptides deduced from ORF A and ORF B, respectively. Second, point mutations in the initiation codons of ORF A and B block the synthesis of the appropriate peptide. Third, radiolabeled peptides A and B could be immunoprecipitated from *in vitro* translation assays containing wild-type *GmENOD40* mRNA. Fourth, peptide B was detected in nodules by Western blotting. Using this technique, Abs directed against peptide A recognized only compounds with higher molecular masses. This observation is in line with previous findings (12) and suggests that peptide A may form large, stable aggregates. In addition, ORFs corresponding to ORF A and B are present in all legume *ENOD40*s studied until now, and the AUG initiation codons of both ORFs and the nearby nucleotide sequences are in the right context for translation initiation (Kozak sequence; ref. 30).

To identify the molecular targets of these unusual short translation products, we have used biotinylated peptides A and B for affinity purification of potential binding proteins from soybean nodule extracts. Using this approach, we found that both peptides bind to the same target protein of 93 kDa (Fig. 5). To identify these proteins, tryptic peptides were generated and subjected to matrix-assisted laser desorption ionization-time-of-flight analyses. Searching of the National Center for Biotechnology Information database with the resulting peptide mass fingerprints identified the 93-kDa proteins as soybean Nodulin100, which is a subunit of SuSy (20).

Symbiotic nitrogen fixation is an energy-demanding process, and the supply of sucrose to the nodule may limit fixation. In addition, the rapidly growing nodule tissue requires imported sucrose for cellulose biosynthesis. SuSy is one of the most

abundant proteins in mature soybean nodules, and its activity increases rapidly during nodule development (20). The enzyme catalyzes the reversible conversion of sucrose and UDP into UDP-glucose and D-fructose. Sucrose, as major end product of photosynthesis, is translocated from the shoots to nodules. The enhanced expression of SuSy during nodule development indicates that SuSy may have an important physiological function in phloem unloading of sucrose. Cleavage of this disaccharide provides nutrients for the bacteroids, energy for the nitrogenase, UDP-glucose for polysaccharide biosynthesis, and carbon skeletons for the assimilation of fixed nitrogen (20, 31). Binding of peptides A and B to SuSy may either regulate enzyme activity or may direct this enzyme to specific intracellular sites. This finding suggests that ENOD40 peptides may contribute to the control of photosynthate use in plants, which is consistent with *in situ* hybridization studies that revealed that *ENOD40* transcripts predominantly occur in the vascular bundles of mature soybean nodules (3).

The recently sequenced *Arabidopsis* genome contains a large number of short ORFs. Such ORFs are usually thought to have no biological significance. Our combined genetic and biochemical data show that small ORFs should receive more attention; otherwise important biological functions may be overlooked.

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