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Phyton (Austria)	**Vol. 28**	Fasc. 1	133–140	20. 7. 1988

Conservation of soybean *(Glycine max)* nodulins and nodulin genes within the tribe *Phaseoleae.*

By

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With 3 Figures

Received June 11, 1987

Key words: Nodulin, nodulin genes, DNA, polypeptides, *Glycine max, Phaseoleae.*

Summary

KATINAKIS P. & MADRZAK C. 1988. Conservation of soybean *(Glycine max)* nodulins and nodulin genes within the tribe *Phaseoleae.*–Phyton (Austria) 28 (1): 133–140, with 3 figures. – English with German summary.

Using immunological techniques, we demontrated that only a small number of soybean nodule-specific polypeptides are antigenically related to nodule polypeptides from legumes of the trible *Phaseoleae.* Genomic DNAs from these plants were also searched for sequences homologous to characterized soybean nodulin genes. Hibridization studies suggest that some of these genes may have diverged extensively in evolution.

Zusammenfassung

KATINAKIS P. & MADRZAK C. 1988. Konstanz der Noduline und Nodulingene der Sojabohne *(Glycine max)* innerhalb des Tribus *Phaseoleae.* – Phyton (Austria) 28 (1): 133–140. – Englisch mit deutscher Zusammenfassung.

Mittels immunologischer Techniken wird gezeigt, daß nur eine geringe Zahl der knöllchenspezifischen Polypeptide von Sojabohne ähnliche antigene Eigenschaften besitzt wie die Polypeptide der Knöllchen von Leguminosen aus der Tribus *Phaseoleae.* DNA aus dem Genom dieser Pflanzen wurde auf Sequenzen, die die Nodulingene der Sojabohne charakterisieren, geprüft. Hybridisierungsversuche legen nahe, daß sich einige dieser Gene während der Evolution stark auseinanderentwickelt haben.

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Introduction

The symbiotic assocoation of *Rhizobium* with legumes results in the development of specialized structures, called root nodules, in which the atmospheric nitrogen is converted to ammonium (VERMA & NADLER 1984). The development of this symbiosis requires the coordinate expression of both bacterial and plant genes. A number of host plant gene products, termed nodulins, induced following infection of legumes by *Rhizobium* spp., have been identified in *Glycine max* (LEGOCKI & VERMA 1980), *Pisum sativum* (BISSELING & al. 1983) and *Medicago sativa* (LANG-UNNASCH & AUSUBEL 1985, VANCE & al. 1985). The best known of such plant gene products are leghemoglobins (VERMA & NADLER 1984), nodulin-35, a nodule-specific uricase (BERGMANN & al. 1983) and a nodule-specific glutamine-synthetase (CULLIMORE & MIFLIN 1984). The function of the other nodulins is not known as yet. Recently, three *G. max* nodulin genes, namely nodulins 23, 24 and 35 have been isolated and their sequences have been determined (KATINAKIS & VERMA 1985, MAURO & al. 1985, NGUYEN & al. 1985). It has been suggested that there may be two distinct groups of nodulins: C-nodulins that are conserved among legumes and S-nodulins that are species specific (VERMA & NADLER 1984). Recent studies have demonstrated that in addition to leghemoglobin, several other nodule-specific polypeptides from *M. sativa* nodules appear to be conserved in a number of taxonomically distant legumes (LANG-UNNASCH & AUSUBEL 1985, VANCE & al. 1985). Furthermore, extensive cross-hybridization of *G. max* leghemoglobin sequences with genomic DNA from various legumes has also been observed (BRISSON & al. 1982).

In this report, we present evidence from immunological and molecular hybridization studies, suggesting that *G. max* nodulin gene products and sequences have partially diverged with the tribe *Phaseoleae*.

Materials and Methods

Plants were grown as discribed by VERMA & al. 1974. Nodules formed, as a result of soybean *(Glycine max)* (L.) MERR., cv. Price), inoculation with *Rhizobium japonicum* strain 61A76, *Phaseolus vulgaris* L. inoculation with *Rhizobium phaseoli* and *Vigna sinensis* (L.) SAVI ex HESSK. inoculation with *Rh. japonicum* strain 110, were harvested three weeks after inoculation. Nodules were frozen in liquid nitrogen.

Plasmid and genomic DNAs were prepared as previously described (KATINAKIS & VERMA 1985). DNA digested with restriction endonucleases (Boehringer, Mannheim) was subjected to electrophoresis on agarose gels and transferred to Gene Screen (New England Nuclear). The following nodulin genes were used as probes: nodulin-24 cDNA, nodulin-35 cDNA, leghemoglobin cDNA and EcoR-HindIII cloned genomic fragment of nodulin-23 gene, which contain the intron, most of the coding, and 5′ end flanking sequences. The primary structure of the aforementioned genes has been published (KATINAKIS & VERMA 1985, MAURO & al. 1985, NGUYEN & al. 1985). The inserts were isolated from agarose gels, labelled with (^{32}P)d CTP (specific activity

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3000 Ci/mmol; 1C = 37GBq; Amersham) as previously described (KATINAKIS & VERMA 1985).

Nodule and root proteins extracted (LEGOCKI & VERMA 1980) from different legumes were run on 7.5–15% (W/V) polyacrylamide sodium laurylsulphate (SDS) gradient gels and transfereed to nitrocellulose (TOWBIN & al. 1979). Filters were incubated with a 1:50 dilution of soybean nodule-specific immune serum which had been preadsorbed with root extracts (STANLEY & al. 1986) and then with (^{125}I) labelled protein A (Pharmacia) as described by FORTIN & al. 1985. Filters were dried and autoradiographed.

Results

A number of nodule-specific polypeptides from the soybean- *R. japonicum symbiosis* have been identified using antisera raised against nodule specific soluble proteins (LEGOCKI & VERMA 1980). These antibodies were used to investigate the presence of common antigenic determinants among nodulins within the tribe *Phaseoleae*. As shown in the immunoblot (Fig. 1, lanes P and V) polypeptides from *Vigna* and *Phaseolus* effective

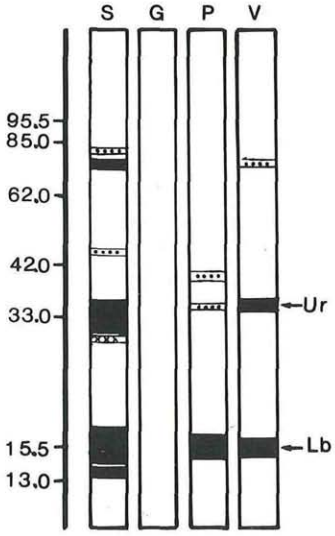

Fig. 1: Cross-reactivity of soybean nodule-specific antiserum with effective nodule proteins of various legumes of the tribe *Phaseolae*. Polypeptides extracted (30 μg) from nodules of soybean (S), *Vigna* (V), *Phaseolus* (P) and uninfected soybean roots (G) were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose and probed with the nodule-specific immune serum. Antigen-antibody complexes were detected using (^{125}I) protein A and autoradiography. Lb, leghemoglobin; Ur, uricase II. Protein mol. wt., markers (×1000) are indicated. Relative intensities of bands in the autoradiograph are indicated by shading: black > ××× > dotted > blanc.

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nodule extracts, cross-react with soybean nodule-specific serum. No reaction was observed with proteins from uninfected soybean root extracts (Fig. 1, lane G), while a number of cross-reacting polypeptides were detected from effective soybean nodules (Fig. 1, lane S). The most prominant protein common in all plant examined is leghemoglobin (as indicated by the arrow [Lb] in Fig. 1). It is also likely that uricase II (a 35Kda protein, indicated by the arrow in [Fig. 1] is one of the common immunoreacting polypeptides. Uricase enzyme activity has also been detected in *Vigna* nodules (SHELP & al. 1983).

In view of the partial conservation of nodule-specific polypeptides among members of the tribe *Phaseoleae,* it was of interest to investigate to what extent sequences homologous to soybean nodulin genes are conserved. To ecamine this, DNA was extracted from soybean, *Vigna* and *Phaseolus* embryonic axes, digested with EcoR1 restriction endonuclease, separated on agarose gels and transferred to Gene Screen. Four identical blots were hybridized separately to various probes derived from characterized soybean nodulin genes (see Material and Methods). The hybridization conditions

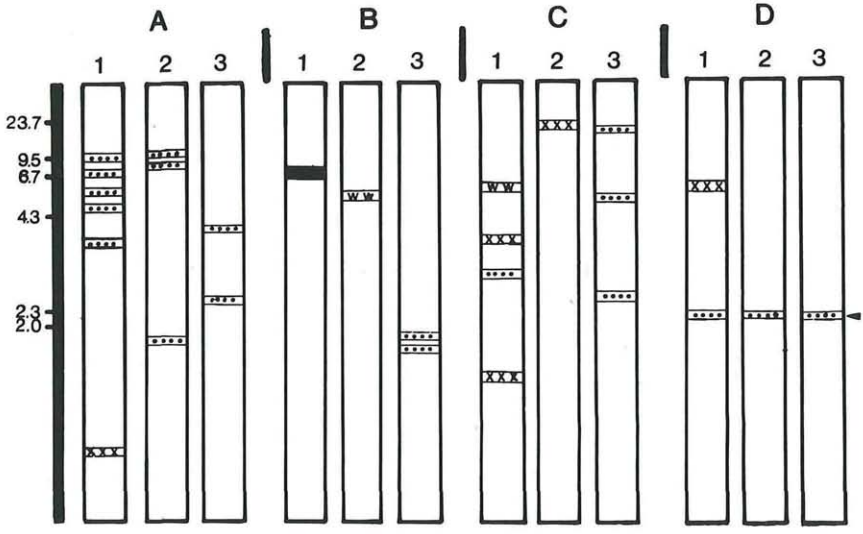

Fig. 2: Hybridization of soybean nodulin genes to DNAs from members of the tribe *Phaseoleae.* DNA (10 μg each) isolated from *Glycine max.* (1), *Vigna* (2) and *Phaseolus* (3) were digested with EcoRI, electrophoresed in 1% (W/V) agarose and transferred to GeneScreen. Four identical blots were prepared and each filter was hybridized to (^{32}P)-labelled leghemoglobin (A), nodulin-24 (B), nodulin-35 (C), a genomic cloned fragment of nodulin-23 gene (D). Bacteriophage lambda HindIII fragments were used as molecular weight markers. Sizes are shown in kb. Relative intensities of bands in the autoradiograph are indicated by shading as in Figure 1.

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employed were of high stringency and would allow the detection of sequences with a relatively high degree of homology (MANIATIS & al. 1982). The autoradiographic image shown in Fig. 2 demonstrated that, in addition to leghemoglobin (Fig. 2, panel A), sequences homologous to nodulins 24 and 35 genes are also present in the genome of the other species (Fig. 2, panels Band C). However, sequences homologous to nodulin-25 gene were absent (Fig. 2, panel D). The observed hybridizing band (as indicated by the arrow in Fig. 2, panel D) common in all plant genomes was not detected when the nodulin-25 cDNA or the sequences flanking the 5′-end of nodulin-25 gene were used as probes. Thus the cross-hybridizing bands may represent the intron sequences present in the probe used.

DNA sequence analysis has shown that soybean nodulin-24 gene has an unusual intron-exon arrangement, comprising of three tandemly repeating units which are located within a 1.3 kb HaeIII fragment (KATINAKIS & VERMA 1985). Since the degree of sequence relatedness existing in specific regions of the genomes of related organisms can be measured by hybridizing radiolabelled cloned DNA to Southern blots of restriction digests of their

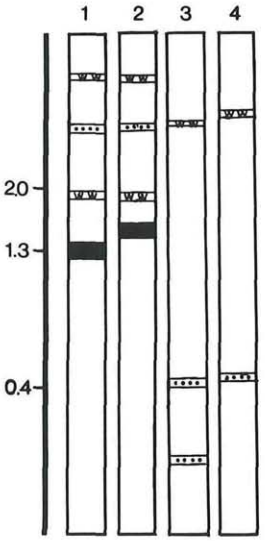

Fig. 3: Southern blot of soybean (1), *Glycine soya* (2), *Vigna* (3) and *Phaseolus* (4) genomic DNA hybridized to one of the repeating units of nodulin: 24 gene. DNAs (10 μg each) from all indicated plants were digested with EcoRI and HaeIII, electrophoresed in 1.5% (W/V) agarose gel, transferred to GeneScreen and hybridized to a cloned repeating unit of nudulin-24. pBR322 HinfI fragments were used as molecular weight markers. Sizes are shown in kb. Relative intensities of bands in the autoradiograph are indicated by shading as in Figure 1.

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genomic DNA (NEI & TAJIMA 1981). It was of interest to determine whether the 1.3 kb HaeIII fragment is conserved within the tribe *Phaseoleae*. As shown in Fig. 3, when one of the cloned repeating units (KATINAKIS & VERMA 1985) was used as a probe, hybridizing bands of different size and intensities were detectable in all plant genomes examined. Interestingly, a hybridizing band of different size was observed even in the closest ancestor of the soybean, *Glycine soya* (Fig. 3, lane 2). However, the 1.3 kb fragment appears to be conserved in all North American soybean varieties (cv. Prize, cv. Williams, cv. Harcor) examined (data not shown). These differences may represent base substitutions at the restriction site(s) or insertion(s) near the site. Furthermore, differences in band intensities can be explained on the basis of gene dosage among the various plants genomes. Copy number experiments revealed the presence of 10–15 copies of the 1.3 kb HaeIII fragment in the genomes of both *G. max* and *G. soya,* while the hybridizing fragment(s) in the genomes of the other plant species appear to be present in 1–2 copies.

Discussion

It has been proposed that some nodulins, C-nodulins, may be serve a specific role in all legumes (VERMA & NADLER 1984). If so, it would be expected that they would share common antigenic determinants. This study has demonstrated that, in addition to leghemoglobin, a number of soybean nodule-specific polypeptides are also present in the nodules of *Vigna* and *Phaseolus* as judges by their immunological cross-reactivity (Fig. 1). Antigenically-related nodule-specific proteins from *M. sativa* have also been reported across a diverse range of legumes (VANCE & al. 1985), thus supporting the hypothesis for the occurence of C-nodulins. It is nevertheless evident from our immunological studies (Fig. 1) and those of other investigators (LANG-UNNASCH & AUSUBEL 1980, VANCE & al. 1985), that the majority of nodulins of various legumes do not appear to share any common antigenic determinants. This can be explained on the basis of species-specific nodulins, as previously suggested (VERMA & NADLER, 1984) or it may reflect a high degree of divergence between closely-related species. A high divergence can account for the low level of cross-reactivity between *Lupinus* and soybean leghemoglobins (VANCE & al. 1985). Although both species contain functional leghemoglobin proteins, the proteins differ at about half of their amino-acid positions (LEHTOVAARA & al. 1980).

Our hybridization data (Fig. 2) show that sequences homologous to soybean nodulin genes are also detectable in *Vigna* and *Phaseolus*.

Whether these genes are functional is not known. Hybridization studies failed to detect nodulin-23 and -24 sequences in polysomal RNA from *Phaseolus* (FULLER & VERMA 1984). However, this does not rule out the possibility that these genes are expressed at a low level, not detectable by

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the technique used. The absence of sequence homologous to the coding region of nodulin-25 gene (Fig. 2, pane D) as well as the observed polymorphism of the restriction sites encompasing the 1.3 kb HaeIII fagments even between *G. max* and *G. soya* (Fig. 3, lanes 1 und 2) suggest that some of the nodulin genes may have diverged relatively late in the evolution of legumes. Divergence (15%) of genomic DNA sequences among members of the tribe *Phaseoleae, G. max* and *P. vulgaris,* have also been reported for leghemoglobin genes (LEE & VERMA 1984). Furthermore, hybridization studies indicated that organelle DNA from taxonomically distant legumes appears to have a high degree of devergence (PALMER & THOMPSON 1982).

Acknowledgments

The authors are grateful to Prof. D. P. S. VERMA, Dept. of Biology, McGill University for his support and for making available laboratory facilities during the course of these studies.

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Zeitschrift/Journal: Phyton, Annales Rei Botanicae, Horn

Jahr/Year: 1988

Band/Volume: 28_1

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Artikel/Article: Conservation of soybean (Glycine max) nodulins and nodulin genes within the tribe Phaseolae. 133-140