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Widespread Occurrence of the Homologues of the Early Nodulin (*ENOD*) Genes in *Oryza* Species and Related Grasses

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Eighty accessions representing 23 species from the genus *Oryza* were examined for the presence of homologues of early nodulin (*ENOD*) genes. Southern analyses indicated a widespread distribution of homologues of *ENOD* genes across all the genomes of rice as well as other monocots. The degree of cross-hybridization of the legume *ENOD* genes with sequences in the genomes of various species, as revealed by hybridization differentials measured in terms of signal intensities, however, suggests that the homologues of *ENOD* genes are conserved to varied extents in different *Oryza* species. The presence of homologues of *ENOD* genes in a wide variety of plant species denotes that the biological functions of early nodulins may be diverse, and not restricted to nodule organogenesis alone. The fact that *ENOD* gene homologues exist widely both in dicots and monocots provides evidence that these homologues have arisen from a common ancestral plant.

Nitrogen is the mineral element that most frequently limits plant productivity, and there is always a need to supplement the crop with additional nitrogen in the form of fertilizer for attaining enhanced crop production. Application of inorganic nitrogen fertilizer though helps in attaining greater yields, its continuous use leads to environmental degradation. Certain plants, such as legumes, that fix nitrogen symbiotically have a built-in supply of reduced nitrogen, which allows them to meet their nitrogen demands independent of nitrogen levels in soil and therefore without the monetary, energy or environmental costs associated with the production and use of nitrogen fertilizer.

The symbiotic association between leguminous plants and rhizobia results in the formation of root nodules, in which the rhizobia reside and fix atmospheric nitrogen as ammonia. All steps of nodule development involve the expression of nodule-specific plant genes (1, 2). During the process of rhizobial infection and subsequent early stages of nodule development, several genes (denoted as early nodulin genes, *ENOD* genes) are expressed in a tissue-specific manner at the site of rhizobial interaction leading to the accomplishment of crucial processes involved in infection and initiation of nodule ontogeny in homologous legume plants.

Rice is the most important staple food for more than 2.4 billion people worldwide. There is much interest recently in determining whether nitrogen-fixating capability could be transferred to a monocot such as rice to render the crop self-reliant in its nitrogen requirements (3, 4 & references therein). One possibility of transferring nitrogen-fixing capability to rice is by developing a rice plant possessing an ability to form enodosymbiotic associations with *Rhizobium* (5). The possibility of extending the host range of rhizobia to nonlegumes was motivated by the discoveries that *Parasponia* forms nitrogen-fixing nodules with *Rhizobium* (6, 7) and that some other nonlegumes like oilseed rape and rice exhibited ability to develop nodule-like structures/hypertrophies, albeit at low frequencies, in response to rhizobial inoculation (3, 8-10 & references therein). Moreover, some nonleguminous plants including rice were found to have the ability to perceive lipochitooligosaccharide nodulation signal molecules (Nod factors) produced by rhizobia (11, 12). These findings suggest that nodule formation programs are conserved, at least partially, in nonlegumes. Inherent nodulation potential of nonlegumes can probably be

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attributed to the existence of homologues of so-called *ENOD* genes in these plants.

Investigations of legume symbioses have unveiled a number of critical genetic components in legumes that are important for the accomplishment of symbioses, but presence of these components has not been assessed in the members of monocotyledonous plants such as rice. In our attempts to determine the genetic predisposition of rice towards rhizobial infection, we initiated investigations on identification and characterization of homologues of *ENOD* genes in rice. In this paper we present evidence for a widespread occurrence of homologues of *ENOD* genes in various rice species across different genomes, and related monocots.

MATERIALS AND METHODS

Plant materials. The material comprised of 80 rice accessions belonging to 23 *Oryza* species, 6 related genera, 2 cultivated monocots (maize and sugarcane) and 1 dicot (soybean) (Table 1). Green leaves of these plant samples were obtained from the Genetic Resource Center and the Plant Breeding, Genetics and Biochemistry Division, International Rice Research Institute.

Probes. Polymerase chain reaction (PCR) generated nodule-specific cDNAs of *PsENOD*5, *PsENOD*12 and *PsENOD*14 from *Pisum sativum* L. cv. Sparkle (13) and subtracted cDNAs of *GmENOD*2 (*GmN234*), *GmENOD40* (*GmN36b*), *GmENOD*55 (*GmN315*), *GmENOD70* (*GmN70*) and *GmENOD*93 (*GmN93*) prepared from nascent nodules of *Glycine max* L. cv. Akisengoku (14) were used as probes in the present study.

DNA extraction, digestion and Southern blotting. Healthy green leaves from actively growing plants at the tillering stage were collected in liquid nitrogen. High molecular weight genomic DNA was prepared from fresh leaves (~10 g) according to the method of Dellaporta *et al.* (15) with minor modifications. The quality and quantity of DNA was determined both spectrophotometrically, as well as visually by ethidium bromide staining on agarose gels.

DNA (8 μg) from each sample was digested with *Dra*1, electrophoresed overnight in a submerged horizontal agarose gel (0.9%) at 30 V in 1x TAE buffer (40 mM Tris acetate; 1 mM EDTA; pH 8.0) and Southern blotted onto Hybond N^+ nylon membrane following alkaline transfer protocol as detailed by the manufacturer (Amersham, U.K.). Six identical sets of Southern blots, each representing 89 plant samples, were made and each set of blots was reprobed 3-4 times after stripping. cDNAs of *ENOD* genes were labeled by random priming with ^{32}P-α-dCTP according to the manufacturers' instructions (Amersham, U.K. and Promega, U.S.A.) and used as probes. Hybridization was carried out in a solution containing 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.5), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.5% SDS and 100 μg ml^{-1} denatured salmon sperm DNA at 58-60°C for 16-18 h (16). The membranes were washed twice for 15 min each in 3× SSC (1× SSC is 0.3 M NaCl, 0.03 M sodium citrate; pH 7.0), 0.5% SDS at 55°C, and then in 2× SSC, 0.1% SDS at 60°C two times for 15 min each. The membranes were autoradiographed for 2-4 days.

Signal quantification. Differences in the hybridization signal obtained on autoradiographs were measured semi-quantitatively by scanning densitometry (Molecular Dynamics densitometer, Sunnyvale, CA, U.S.A.) according to Aggarwal *et al.* (17). The total hybridization signal intensity of band(s) in individual lanes on the autoradiographs was calculated in optical density units in volume integration mode after subtracting the background value. Subsequently, for the estimation of hybridization differentials, the values obtained with a particular *ENOD* probe were normalized relative to the total hybridization signal intensity of the bands derived from the soybean genomic DNA probed with the same *ENOD* cDNA.

RESULTS AND DISCUSSION

DNA transfer blot analysis of *Oryza* and related genera including maize and sugarcane genomic DNA probed with legume *ENOD* cDNAs revealed that homologues of *ENOD* genes are widely distributed across all genomes of *Oryza* and other plant species tested (Figs. 1-3, Table 1).

The *Dra*1-digested DNA of *Oryza* species probed with *ENOD2, ENOD5* and *ENOD12* cDNAs revealed the homologue-banding profiles more distinctly on DNA transfer blots than *ENOD14, ENOD40, ENOD55, ENOD70* and *ENOD93* probes. The differential hybridization profiles generated by probing with *ENOD* cDNAs, nevertheless, demonstrated that the majority of the variation in general was found to be between accessions from different species. On the other hand, except in a few species, the variation between accessions within a species was found to be marginal. These findings evidence a closer genetic relatedness within a species, irrespective of their geographical distribution, with respect to the distribution of homologues of *ENOD* genes.

Stringency of the posthybridization washing regime (2× SSC, at 60°C) of DNA transfer blots employed in the present study favors the visualization, on the autoradiographs, of DNA sequences with approximately 50-65% homology to the *ENOD* probes used (17). In the present study, of all the *ENOD* probes employed, *ENOD40,* a critical gene responsible for cortical cell divisions leading to the initiation of nodule development in legumes (1), produced relatively weak hybridization signals in all genomes of *Oryza* species and related monocots tested (Fig. 1, Table 1). Despite this, characterization of the *ENOD40* homologue from *Oryza sativa* revealed about 50% nucleotide identity to the soybean *ENOD40* (Kouchi *et al.*, submitted). Furthermore, analysis of an another homologue, *ENOD93* isolated from *O. sativa,* which exhibited hybridization signal of low intensity when probed with the legume *ENOD93* cDNA (Table 1), revealed a homology of more than 60% to soybean *ENOD93* (18). These findings evidenced that the hybridizing fragments are indeed homologues of *ENOD* genes. The findings also demonstrated that the employed stringency-wash conditions enabled the visualization of the homologues having a minimum of 50-60% identity to the *ENOD* probes used.

Though all species tested exhibited the DNA bands that hybridized with the *ENOD* probes, intensity of the bands generated in response to a particular probe varied depending on the species (Table 1). Semi-quantitation of hybridization signals revealed that in comparison to the rest of the species, relatively more

TABLE 1

Semiquantitation of Hybridization Signals Obtained in Various Genomes of *Oryza* Species and Related Monocots after Probing with Legume *ENOD* cDNAs

Serial No.	Species	Genome	Accession	Origin	% Hybridization signal (relative to soybean)							
					ENOD2	*ENOD5*	*ENOD12*	*ENOD14*	*ENOD40*	*ENOD55*	*ENOD70*	*ENOD93*
1	*O. sativa*	AA	IR-31917	IRRI								
2			IR-56	IRRI								
3			IR-64	IRRI								
4	*O. rufipogon*	AA	105908	Thailand								
5			105909	Thailand								
6			105910	Thailand								
7			106412	Vietnam								
8			106423	Vietnam								
9	*O. longistaminata*	AA	103886	Tanzania								
10			103890	Senegal								
11			103902	Tanzania								
12	*O. barthii*	AA	101937	Senegal								
13	*O. nivara*	AA	103407	Sri Lanka								
14			105721	Cambodia								
15			106185	India								
16	*O. glumaepatula*	AA	100969	Suriname								
17	*O. perennis*	AA	104823	Thailand	*							
18	*O. punctata*	BB	103896	Tanzania								
19			104064	Nigeria								
20			105690	Kenya								
21			105980	Cameroon								
22	*O. punctata*	BBCC	100884	India								
23			101409	Ghana								
24			104975	Kenya								
25	*O. officinalis*	CC	100896	Thailand								
26			101116	Philippines								
27			101399	Vietnam								
28			105100	Brunei								
29			105220	Indonesia								
30			100176	via CRRI, Ind.								
31	*O. rhizomatis*	CC	103421	Sri Lanka								
32			105448	Sri Lanka								
33			105449	Sri Lanka								
34	*O. eichingeri*	CC	101424	Uganda		*						*
35			105181	Uganda								
36			105182	Uganda								
37			105408	Sri Lanka								
38			105413	Sri Lanka								
39	*O. minuta*	BBCC	101089	Philippines								
40			101141	Philippines								
41			103876	Philippines								
42			105253	Philippines								
43	*O. alta*	CCDD	100888	via CRRI, Ind.								
44			100952	via CRRI, Ind.								
45			100967	Suriname								
46			105143	Guyana								
47	*O. latifolia*	CCDD	100168	Costa Rica								
48			100914	Mexico								
49			100955	??								
50			103787	Colombia								

TABLE 1—*Continued*

Serial No.	Species	Genome	Accession	Origin	% Hybridization signal (relative to soybean)							
					ENOD2	*ENOD5*	*ENOD12*	*ENOD14*	*ENOD40*	*ENOD55*	*ENOD70*	*ENOD93*
51	*O. grandiglumis*	CCDD	105155	Brazil								
52			105157	Brazil								
53			105560	Brazil								
54			105669	Brazil								
55	*O. malamphuzhaensis*	BBCC ?	105223	India								
56			105328	India								
57			105329	India								
58	*O. ridleyi*	HHJJ	100820	Thailand								
59			100821	Thailand								
60			106028	Thailand								
61			105973	Indonesia								
62	*O. longiglumis*	HHJJ	105146	Indonesia								
63			105147	Indonesia								
64			105148	Indonesia								
65			105562	Indonesia								
66	*O. australiensis*	EE	100882	via CRRI, Ind.								
67			103318	Australia			*					
68			105269	Australia								
69			105272	Australia							*	
70	*O. brachyantha*	FF	101232	Sierra Leone								
71			94-10482	via CRRI, Ind.				*	*	*		
72	*O. granulata*	GG	100879	India								
73			102118	Thailand								
74			104503	Malaysia								
75			106449	India								
76			104986	via CRRI, Ind.								
77	*O. meyeriana*	GG	wsp-90-5	??								
78			106473	Philippines								
79			106474	Philippines								
80	*O. indandamanica*	GG	105694	India								
81	*Portaresia corctata*	.....	104502	Bangladesh								
82	*Leersia tisseranti*	.....	101384	New Guinea	*	*		*				
83	*Leersia perrieri*	.....	105164	Madagascar								*
84	*Rhynchoryza subulata*	.....	100913	Argentina					*	*		
85	*Hygroryza aristata*	.....	105457	Sri Lanka							*	
86	*Chikusichloa aquatica*	.....	106186	Japan			*					
87	Maize	.....	Local cultivar	Philippines								
88	Sugarcane	.....	Local cultivar	Philippines								
89	Soybean (control)	.....	Clark	Philippines								

Note. Hybridization signal strengths: 100% (■); 51–75% (▩); 26–50% (▤); less than 25% (▭). Asterisks depict the plant species in the *Oryza* complex and in the related genera that gave relatively the strongest hybridization signals with respective probes. Densitometric readings were scored from the autoradiographs showing the hybridization signals that did not saturate the densitometer detector response. Serial numbers of the species correspond to the numbers denoted over the lanes in the Southern blots represented in Figs. 1–3.

intense bands were produced with *ENOD2* probe in the case of DNA of *O. sativa, O. rufipogon, O. longistaminata, O. nivara* and *O. perennis* species belonging to AA genome, with *ENOD14, ENOD40* and *ENOD55* in *O. brachyantha* of FF genome and with *ENOD70* in *O. australiensis* of EE genome (Fig. 2, Table 1). Also, compared to that in other *Oryza* species, the bands visualized by *ENOD5* were found to exhibit stronger signal in *O. punctata* (both BB and BBCC), *O. eichingeri* (CC), *O. minuta* (BBCC) and *O. malamphuzhaensis* (BBCC) while with *ENOD12* it was in *O. australiensis* (EE) and *O. brachyantha* (FF) and with *ENOD93* in *O. sativa, O. rufipogon* and *O. nivara* (AA), and *O. rhizomatis* (CC), *O. eichingeri* (CC), *O. minuta* (BBCC), *O. malamphuzhaensis* (BBCC) and *O. latifolia* (CCDD). Among the related genera of *Oryza,* DNA bands of higher signal strength were generated with *ENOD2* probe in *Leersia tisseranti, L. perrieri* and *Rhychoryza subulata* while with *ENOD5* and *ENOD14* only in *L. tisseranti* (Fig. 3, Table 1). On the other

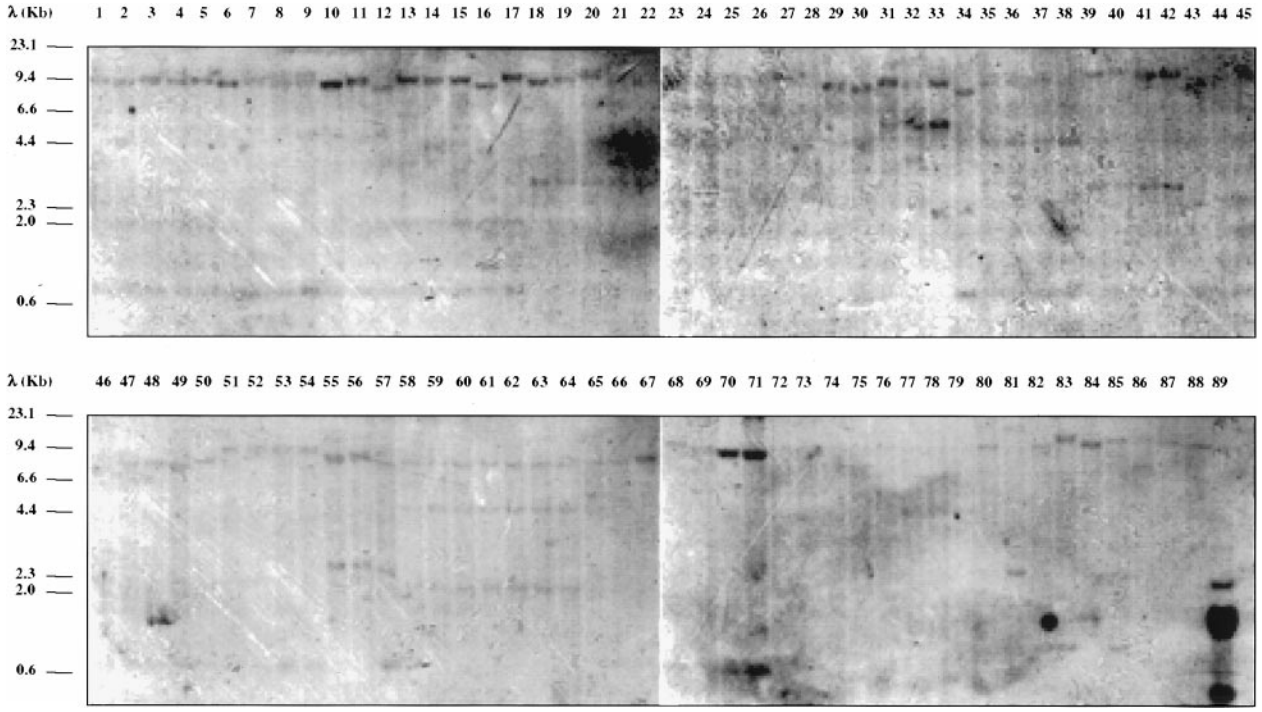

FIG. 1. Southern blots of *DraI*-digested DNA of 89 representative *Oryza* species and other taxa hybridized with ^{32}P-labeled *ENOD40* cDNA probe. Numbers represented over the lanes correspond to the serial number of the species denoted in Table 1.

hand, probing with *ENOD12, ENOD55, ENOD70* and *ENOD93* displayed intense hybridization signals with *Chikusichloa aquatica, R. subulata, Hygroryza aristata* and *L. perrieri,* respectively. With *ENOD40,* however, relatively more prominent bands were visualized in *L. perrieri* and *R. subulata.* It is well established that the DNA sequences that are more homologous to the probes form stronger hybrids and exhibit the bands with higher intensity on autoradiographs than those that are less related. In the present study, the apparent contrasts in the intensities of the bands that are visualized by the *ENOD* probes suggest that the homologues of *ENOD* genes are conserved to varied extents in different *Oryza* species and related genera. It is likely that in the species, the hybridizing fragments which displayed greater intensity upon hybrid-

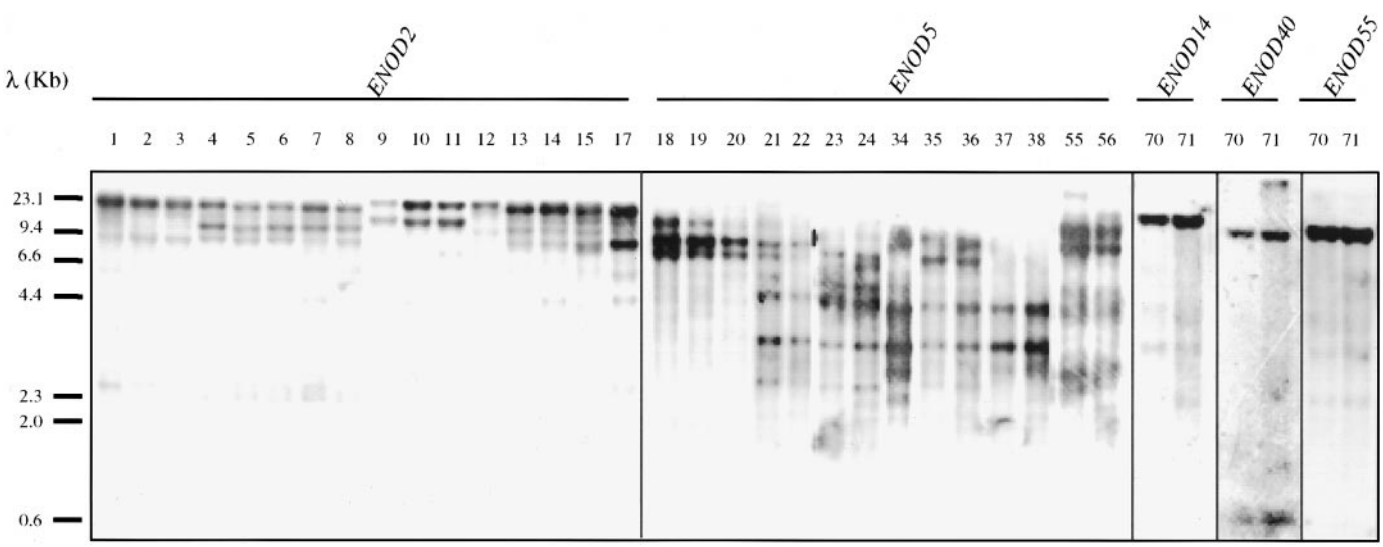

FIG. 2. Southern blots of *DraI*-digested DNA of *Oryza* species that gave strong hybridization signals with ^{32}P-labeled *ENOD* cDNA probes. Numbers represented over the lanes correspond to the serial number of the species denoted in Table 1.

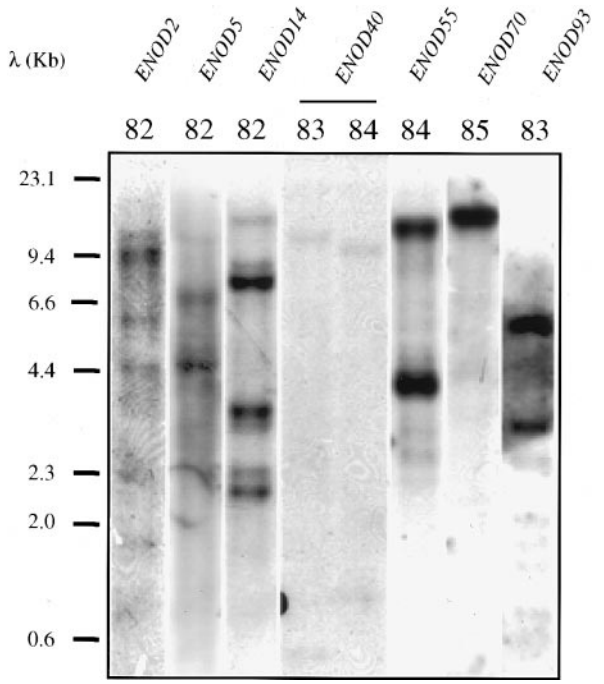

FIG. 3. Southern blots of *DraI*-digested DNA of monocotyledenous genera related to *Oryza* species that gave strong hybridization signals with ^{32}P-labeled *ENOD* cDNA probes. Numbers represented over the lanes correspond to the serial number of the species denoted in Table 1.

ization with the *ENOD* probes on DNA gel blots represent the *ENOD* homologues which are more conserved than in those which displayed the bands of lower intensities.

Despite the general perception that the *ENOD* genes are exclusively induced during nodule organogenesis (19), some of these genes are also expressed, albeit to a lesser extent, in non-symbiotic organs of legumes (14, 20–23). In addition, recent studies revealed the existence of homologues of *ENOD40* even in a nonlegume such as tobacco (24). In the present study, cross-hybridization of legume *ENOD* genes with sequences in the genomes of *Oryza* species and other related genera suggest that the *ENOD* gene homologues are widely dispersed across monocots as well, and possibly ubiquitous in all plants. The expression of the homologues of the *ENOD* genes in various organs of legume plants, as well as the presence of *ENOD* gene homologues in a wide variety of plant species suggest that the biological functions of early nodulins may be diverse, and not restricted to nodule organogenesis alone. Though the precise functions of the *ENOD* gene products in legume nodule organogenesis are yet to be determined, these early nodulins fall into broad general categories such as prolin-rich cell wall proteins (ENOD2, ENOD5 and ENOD12), putative metal-binding proteins (ENOD14, ENOD55), auxin modulators (ENOD40) and membrane sulfate transporters (ENOD70) (25 & references therein). Characterization of homologues of *ENOD40* (Kouchi *et al.*, submitted) and *ENOD93* (18) from rice revealed that they encode peptides that are considerably homologous to the proteins encoded by the corresponding genes in legumes, but their expression is not associated with symbiotic interactions. Thus it is likely that the products of *ENOD* gene homologues perform more general functions in controlling growth and development of plants. In legumes, however, similar to that of the symbiotic hemoglobin genes (26), the nodule-specific alleles of various *ENOD* genes might have apparently evolved as a result of gene divergence/modification in order to meet the specific requirements of nodule organogenesis. The fact that *ENOD* gene homologues exist widely both in dicots and monocots probably suggest that these homologues are derived from corresponding ancestral *ENOD* gene homologues in progenitor plants, and the subsequent evolution of the *ENOD* genes with nodule-specific symbiotic functions resulted after the separation of dicots from monocots.

The research on the *Rhizobium*-legume symbiosis has revealed that the host plant possesses a genetic program for the development of root nodule, a niche for rhizobial inhabitation, that is activated by signal molecules such as Nod factors produced by the microsymbiont (27 & references therein). It is unlikely that a monocot plant such as rice would possess the complete complement of genes or genetic programs involved in the nodule ontogeny program that could be induced by rhizobial strains. However, a reason for optimism is that rice, although do not develop symbiotic association with rhizobia, is able to enter into symbiotic associations with mycorrhizal fungi (28). Genetic links between the processes involved in nodulation and arbuscular mycorrhiza have been found in legumes (29, 30). Studies on nodulation mutants of pea have demonstrated that the early nodulin genes *ENOD2*, *ENOD11*, *ENOD12* and *ENOD40* which control initial steps of nodulation also govern early stages of mycorrhiza development (31). Thus, as rice is able to form symbiotic associations with mycorrhizal fungi, and since the formation of such an association of mycorrhizal fungi with legumes is mediated by *ENOD* genes, it can be inferred that at least some of the genetic machinery required to promote endosymbiosis with rhizobia likely exist and function in rice. Recent studies revealed that indeed rhizobial Nod factors are able to induce the expression of legume *ENOD12* promoter in rice, thus strongly suggesting that at least a portion of the signal transduction machinery important for legume nodulation exist in rice (12). The present study demonstrated that homologues of *ENOD* genes are conserved, probably to varying extents, in all *Oryza* species. This finding is reinforced by the characterization of two of these homologues, *OsENOD40* (Kouchi *et al.*, submitted) and *OsENOD93a* (18), from rice which

revealed open reading frames for encoding peptides having significant homologies to legume ENOD40 and ENOD93, respectively. Taken together, these findings suggest that the genetic machinery regulating nodule development in legumes is conserved, at least partially, in rice. It is therefore essential that future studies be extended at the cellular and molecular levels to identify why rhizobia-induced symbiotic responses do not fully occur in rice, in order to contemplate genetically engineering this major cereal crop to form a more intimate endosymbiotic association with rhizobia.

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