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A mutation affecting symbiosis in the pea line Risnod27 changes the ion selectivity filter of the DMI1 homolog

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Abstract

After identifying regions of cDNA conserved between the symbiotic gene *DMI1* of the model species *Medicago truncatula* and the homologous genomic region of *Arabidopsis thaliana*, universal primers were designed from 8 of 12 exons to allow the routine amplification of plant homologs. As an example, the complete homologous sequence from the pea (*Pisum sativum* L.) was amplified and sequenced, although the poorly conserved 5'-end and 5'-flanking region of the gene had to be amplified using a modified TAIL-PCR strategy. The identity of this amplified homolog with the *SYM8* gene was independently confirmed by the presence of a single nucleotide change in the coding sequence of the mutant line Risnod27 (sym8) that cosegregated with the asymbiotic phenotype. Five insertions in pea introns responsible for increasing the total length of *SYM8* by 1443 bp, compared to the *M. truncatula* homolog *DMI1*, belong to known transposon and retrotransposon families of pea and legumes in general. In view of the predicted function of SYM8 as an ion channel, the Risnod27 mutation (His309Tyr) appears to be localized in the selectivity filter domain. This finding confirms the essential role of histidine 309 in the symbiotic function of SYM8 and provides a guide to its ionic specificity. In view of the Risnod27 symbiotic phenotype, we hypothesize that SYM8 does not have identical functions in the transduction of rhizobial and mycorrhizal signals. The variability of the N-proximal region of the known legume homologs of DMI1 suggests an interaction with a variable ligand.

Additional key words: Nod factor, *Pisum sativum*, recognition, *Rhizobium,* TAIL-PCR, universal primers.

Introduction

Symbiosis between host plants of the family *Viciaceae* (legumes) and nodule bacteria (rhizobia) is a representative variation of plant symbiosis with important economic consequences, due to the ability of nodule bacteria to fix atmospheric nitrogen and pass it to the host plant. In parallel to the bacterial determinants of symbiotic ability, the so-called nodulation (*nod, nol* or *noe*) genes (Spaink 2000), the establishment of symbiosis in the host plant is controlled by the complementary host symbiotic genes. Mutations in these genes can interrupt symbiosis formation at a number of stages, chronologically starting from the early responses of root hair curling, calcium spiking (Catoira *et al.* 2000, Walker *et al.* 2000) and infection thread formation through the induction of nodule divisions in the root cortex up to the formation and persistence of symbiotic tissue with intracellularly-located and differentiated bacteria (Häser *et al.* 1992, Voroshilova *et al.* 2001).

Although the mutations affecting symbiosis were originally described in the traditional legume models of agricultural importance like soybean (Carroll *et al.* 1986) and pea (Jacobsen 1984), the technical constraints associated with positional cloning in their complex genomes initiated the turn to the model legumes *Medicago truncatula* Gaertn. and *Lotus japonicus* (Regel) Larsen (Sagan *et al.* 1995, Handberg and Stougaard

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Abbreviations: ARMS - amplification refractory mutation system; PCR - polymerase chain reaction; TAIL-PCR - thermal asymmetric interlaced PCR.
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1992). To date, a significant number of early symbiotic genes of *M. truncatula* as well as their *L. japonicus* orthologs have been characterized, comprising genes coding for receptor kinases (Endre *et al.* 2002, Stracke *et al.* 2002, Radutoiu *et al.* 2003, Limpens *et al.* 2003), Ca^{2+}/calmodulin-dependent protein kinase (Lévy *et al.* 2004), ion channels (Ané *et al.* 2004, Imaizumi-Anraku *et al.* 2005) and transcription factors (Schauser *et al.* 1999, Smit *et al.* 2005).

Since the primary action of rhizobia on the host root is mediated by the final products of nodulation gene activity, denoted as Nod factors and chemically characterized as substituted lipochitooligosaccharides (Spaink 2000), attention has concentrated on the early-blocked plant nodulation mutants which are thought to affect Nod factor perception and signal transduction (Downie and Walker 1999). One of the characterized symbiotic genes that has been shown to act in the early stages is *DMI1* of *M. truncatula* (Ané *et al.* 2004).

In order to prepare a tool for characterizing the genomic sequences of *DMI1* homologs in other legume species, we designed a set of universal PCR primers enabling the fast and robust amplification of homologous regions. A comparison of the published coding sequence of *DMI1* (Ané *et al.* 2004) with that of the *Arabidopsis thaliana* homolog identified conserved nucleotide stretches in *DMI1* that were suitable for primer design, permitting subsequent PCR amplification of the corresponding regions in other plant species.

When applied to *P. sativum*, the set of primers were able to amplify the symbiotic gene *SYM8* (Kneen *et al.* 1994) that has been suggested to be orthologous to DMI1, based on the similarity of scarce phenotypic traits in plant mutants and on the proximity of invariant genomic markers (Kneen *et al.* 1994, Schneider *et al.* 2002, Ané *et al.* 2002). Together with two other early-acting genes *SYM19* and *SYM9*, the *SYM8* gene has been shown to participate in forming endomycorrhizal symbiosis at the stage of epidermis penetration (Provorov *et al.* 2002).

A single nucleotide change identified in the pea line Risnod27 (sym8) linked the amplified homolog to the *SYM8* gene. The rare location of the Risnod27 mutation in the selectivity filter region of the putative ion channel coded by *SYM8* allows hypotheses on the ionic interactions of this molecule having a key role in symbiosis development to be tested.

Materials and methods

Plants: To determine the symbiotic phenotype, the pea (*Pisum sativum* L.) plants were grown hydroponically on N-free nutrient solution according to Škrdleta *et al.* (1984) in a growth chamber at 500 μmol(photon) m^{-2} s^{-1} of photosynthetically active radiation, 16-h photoperiod and day/night temperature of 22/16 ºC as previously described (Novák 2003). Pea cv. Finale and the non-nodulating mutant lines Risnod20, Risnod25 and Risnod27 (Engvild 1987) derived from the cv. Finale with chemical mutagenesis were obtained from Risø National Laboratory, Risø, Denmark. The field pea line Zhodino E900 was obtained from Selgen, Lužany, Czech Republic, while the non-nodulating line R25 induced by γ-ray mutagenesis in cv. Sparkle (Kneen *et al.* 1994) was obtained from the pea germplasm collection of John Innes Institute, Norwich, United Kingdom (registered as line JI3028). The seeds of pea cv. Bohatýr were provided by Osiva Boršov, Czech Republic. The genetic analysis of pea mutants, as well as the recurrent backcrosses for obtaining a cv. Bohatýr nearly-isogenic line harboring a mutation from the non-nodulating line Risnod27, were carried out as described previously (Novák 2003) up to the identification of B_6 non-nodulating segregants.

Microsymbionts: The development of symbiotic root nodules and nitrogen autotrophy were tested with the standard 248 strain of *Rhizobium leguminosarum* bv. *viciae* (Josey *et al.* 1979). Bacterial culture and the inoculation of pea seeds with bacterial suspension were performed as previously described (Novák 2003).

Mycorrhiza development was estimated in plants grown under field conditions (Vestec near Prague, orthic luvisol) in the 2004 and 2005 seasons. The washed roots were fixed with an ethanol-acetic acid mixture (2:1) and stained for arbuscular mycorrhiza with trypan blue according to Demchenko *et al.* (2004). Segments of lateral roots (12 cm) were mounted on microscopic slides and inspected for the presence of intraradical hyphae, arbuscules and vesicles with an Amplival microscope (*Zeiss*, Jena, Germany). The frequency of the occurrence of symbiotic structures per 1-cm root segment was used to quantify mycorrhiza development. At least six plants were estimated for each genotype.

Nucleic acid isolation and reverse transcription: After symbiotic phenotype development 6 weeks after sowing, leaflets from individual pea plants representing 30 - 40 mg of fresh mass were harvested into 1.5 cm^3 Eppendorf tubes, frozen in liquid nitrogen and stored at -70 ºC. Genomic DNA was isolated according to Ellis (1994) with subsequent phenol extraction and ethanol precipitation (Sambrook *et al.* 1989).

Total RNA was isolated from N_2-frozen roots of uninoculated and aseptically grown seedlings using an adapted general technique (Ausubel *et al.* 1989). After the electrophoretic check of integrity and a spectrophotometrical adjustment of concentration, specific mRNA was reverse-transcribed with RevertAid kit (*MBI Fermentas*, Vilnius, Lithuania) under conditions specified by the manufacturer.

Primer design and PCR amplifications: The cDNA sequence of *Medicago truncatula DMI1* (AY497771) and its corresponding genomic sequence (BAC clone

mtH2-54A24, AC140550), the cDNA sequences of *Lotus japonicus CASTOR* and *POLLUX* genes (AB162157 and BAD89020, respectively) as well as the homologous rice genomic (PAC clone P0039A07, AP003235) and cDNA (BAD81710) sequences were obtained from the EMBL DNA database. The homologous *Arabidopsis thaliana* genomic sequence (TAC clone K9P8, AB024032) was obtained from *Arabidopsis thaliana* database (AAtDB), Stanford University, Stanford, USA. Amplification primers were designed, following the general rules, from 21 - 24 bp long stretches having a minimum number of *M. truncatula*/*Arabidopsis* mispairings.

The PCR reaction mixture contained 10 mM Tris-HCl buffer (pH 8.8), 50 mM KCl, 0.08 % *Nonidet*, 1.25 mM $MgCl_2$, 0.15 mM dNTP, 0.2 μM of each primer (*Sigma-Proligo*, Paris, France), 0.1 μg cm^{-3} of template DNA (or cDNA obtained from 1 μg of total RNA cm^{-3}), and 40 U cm^{-3} of recombinant Taq polymerase (*MBI Fermentas*) in a total volume of 0.02 cm^3. Cycling included 34 cycles of 0.5 min (1 min in the 1st cycle) at 93.5 °C, 1 min (3 min in the 1st and 2nd cycles) at a chosen annealing temperature and 2 min (8 min in the last cycle) at 72 °C. For the preparative reactions having an increased reaction volume of 0.05 cm^3, the incubation at the denaturation (93.5 °C) and annealing steps was prolonged by 15 s and five cycles were added. Two preparative reactions were pooled during product purification on a *QIAquick* PCR purification column (*Qiagen*, Valencia, USA). The formation of a fragment of the expected size was checked with agarose gel electrophoresis (Sambrook *et al.* 1989).

The purified PCR fragments were sequenced with the *ABI PRISM BigDye* terminator *v3.1* cycle sequencing kit (*Applied Biosystems*, Foster City, USA). The chain termination reaction (Sanger *et al.* 1977) was performed with the cycle sequencing technique (Murray 1989) according to the manufacturer's protocol. The sequencing products were purified by gel filtration using *DyeEx* spin columns (*Qiagen*). Finally, the sequences were determined with an *ABI PRISM 3100* DNA sequencer (*Applied Biosystems*).

TAIL-PCR: To cover the region of low homology at the 5'-end of *MtDMI1/AtDMI1*, as well as the 5'-flanking region, the thermal asymmetric interlaced PCR (TAIL-PCR) technique of Liu *et al.* (1995) was adapted. While the outer primer presumably contained a high number of mismatches since it was designed from a region of poor homology upstream of the *M. truncatula* gene, two internal primers corresponded to conserved regions. In the primary cycling program, the radically decreased annealing temperature in the interlaced cycles allowed for low-specificity annealing of the outer primer, while the high-stringency cycles restored the selectivity provided by the most distant of the internal primers (Terauchi and Kahl 2000). The program was comprised of 4 stringent cycles (0.5 min at 93.5 °C, 1 min at a chosen T_a, 4 min at 72 °C) followed by one permissive cycle (0.5 min at 93.5 °C, 1 min at 40 °C, 4 min at 72 °C) and by 14 repeated series of two stringent and one permissive cycles. The initial denaturation and annealing steps as well the final extension step were prolonged three times. Selectivity was further restored by the secondary amplification of 20 cycles carried out at a stringent annealing temperature calculated for the outer and the proximal internal primers. The products from the primary reaction served as a template after being diluted 100-fold.

Mutation detection: Purified DNA fragments were subjected to denaturation at 95 °C for 10 min and to hybrid reannealing during gradual cooling from 85 to 25 °C at a rate of 3 °C min^{-1} in the annealing buffer of the *Surveyor* mutation detection kit (*Transgenomic*, Omaha, USA) at a concentration 20 μg cm^{-3}. Heteroduplexes were exposed to CEL-type mismatch-specific *Surveyor* nuclease S for 20 min at 42 °C in *Surveyor* reaction buffer and screened for the presence of restriction fragments with agarose gel electrophoresis.

Computer analysis: Sequence editing, sequence alignment and protein hydrophobicity profiling was carried out with the program *BioEdit* version *7.0.1* (Hall 1999). Homology searches were performed with the BlastN and BlastP programs (srs.ebi.ac.uk/srsbin/cgi-bin/wgetz at the European Bioinformatics Institute). The terminal chloroplast signal peptide was predicted with *ChloroP* prediction software (Emanuelsson *et al.* 1999) as available through the Technical University of Denmark at www.cbs.dtu.dk/services/ChloroP/.

Results and discussion

Phenotypic and genetic characterization of Risnod27: The mutant pea line of interest, Risnod27, was characterized with respect to its symbiotic phenotype and assigned to the locus *SYM8*. All the mutant lines exploited in our work, *i.e.* Risnod20, Risnod25, Risnod27 and R25, exhibited a non-nodulating phenotype both with the standard strain *R. leguminosarum* bv. *viciae* 248 and the local soil population of rhizobia. However, Risnod27 occasionally formed underdeveloped (tiny and white) symbiotic nodules when grown hydroponically under a low water potential.

Risnod27 was found to be allelic with sym8 reference lines Risnod25 and R25 in reciprocal crosses and subsequent tests for F_1 symbiotic phenotype. Mutations in all three sym8 lines were monogenic recessive, consistent with the previous reports for Risnod25 (Novák 2003) and R25 (Kneen *et al.* 1994).

The typical sym8 mutants are known to be blocked early in symbiosis development, as judged from the absence of the root hair curling response, infection thread

formation and *de novo* cortical cell divisions (Markwei and LaRue 1992, Sagan *et al.* 1994, Tsyganov *et al.* 2002), as well as from the block in the synthesis of early symbiosis-specific proteins, the so-called nodulins (Albrecht *et al.* 1998). The defect in calcium spiking placed the *SYM8* gene product functionally upstream of the product of another early-acting gene, *SYM9,* in symbiosis development (Walker *et al.* 2000).

SYM8 is also known to affect the development of endomycorrhizal root symbiosis (Kolycheva *et al*. 1993). Nevertheless, a mycorrhizal phenotype unlike that of two other sym8 mutants inspected was revealed in line Risnod27. While no mycorrhizal structures were detected inside the roots of the Risnod25 and R25 lines, mycorrhiza developed in line Risnod27, albeit to a reduced extent compared to the wild type. While Risnod27 roots contained 14.6 ± 4.8 % of 1-cm root segments positive for arbuscules and 4.2 ± 2.8 % of segments positive for vesicles, the roots of cv. Finale contained 50.7 ± 5.6 % and 27.2 ± 5.4 % of segments with arbuscules and vesicles, respectively. A *SYM8*-independent negative control for mycorrhiza estimates was provided by line Risnod20 that has a mutation in the *SYM19* locus (Kneen *et al.* 1994, Endre *et al.* 2002), and is also reported to be Myc$^-$ (Walker *et al.* 2000).

Table 1. Universal primers used for the amplification of *SYM8* (*PsDMI1*) genomic sequence. The primers correspond to the conserved *M. truncatula* (*Mt*) stretches, as found in the comparison with the *A. thaliana* (*At*) homolog. Only the Ps-marked primers were derived from the *P. sativum* (*Ps*) sequence. The degree of conservancy is quantified by the number of interspecific mispairings. ND - homologous region not detected.

	Denotation	Sequence 5'-3'	Length [n]	Number of mispairings *Mt*/*At*	*Mt*/*Ps*
Forward primers	HD3F	CAAAGACAAGATCTCTCTCTCT	22	6	ND
	A3F	AATCCCAATGACAACAATGGAA	22	6	5
	C2F	TGGTGGTTTAGCGTTGTATGC	21	2	0
	D2bF	ATATGTAGAAGTGGCAGTCCACT	23	0	0
	G1F	ATGGGAGGACATTCTAGGATTTGAG	25	2	1
	H1F	CGCCGTGACATTGATGATATGATCA	25	2	0
	HI5F (Ps)	CTGTGGTAATGGTAAGTAGTCAC	23	ND	0
	I2F	AGGGAAATGCTGTCATTAGGCG	22	3	0
	L0F	TGTGTATTAAGCCAGCAGAGTT	22	5	2
Reverse primers	B2R	GTTTGGCAAGTTTGTATTGTAAA	23	10	2
	C2R	GCATACAACGCTAAACCACCA	20	2	0
	D2bR	AGTGGACTGCCACTTCTACATAT	23	0	0
	G1R	CTCAAATCCTAGAATGTCCTCCCAT	25	0	1
	H1R	TGATCATATCATCAATGTCACGGCG	25	2	0
	HI5R (Ps)	GTGACTACTTACCATTACCACAG	23	ND	0
	I2R	CGCCTAATGACAGCATTTCCCT	22	3	0
	IL2bR (Ps)	AGAGTCCTCCACTGACTCATCT	22	ND	0
	L1R	ACAACAAAAACATCATCAAGGGACCA	26	0	2
	TL1nR	AAGCGCATAAACTGTATAAGTAG	23	14	ND

Table 2. Pairs of universal primers used for the amplification of *SYM8* (*PsDMI1*) genomic sequence and gene junctions. The primers correspond to the conserved *M. truncatula* stretches except for the Ps-marked primers that were derived from *P. sativum* sequence. The position of the 5'-terminal nucleotide of each primer is numbered relative to the translation start in the genomic sequence of the corresponding species. ND - homologous region not detected. A*sterisk* marks primer pairs used in the second round of amplification. T_a - optimized annealing temperature.

Primer combination	*M. truncatula* primer position forward	reverse	fragment length [bp]	*P. sativum* primer position forward	reverse	fragment length [bp]	T_a [°C]	Cycling
HD3F-C2R	-148	1004	1152	ND	1608	1640	60 - 40	TAIL primary
HD3F-B2R	-148	563	711	ND	577	610	55	TAIL secondary
A3F-D2bR	112	1592	1480	163	2266	2103	60	standard
C2F-G1R	984	2259	1275	1588	2949	1361	58	standard
D2bF-H1R	1570	2793	1223	2244	3704	1460	58	standard
G1F-HI5R(Ps)*	2235	3875	1640	2925	4826	1901	60	standard
H1F-I2R	2769	4128	1359	3680	5441	1761	60	standard
HI5F(Ps)-IL2bR(Ps)*	3853	4574	721	4804	5992	1188	60	standard
I2F-L1R	4107	5401	1294	5420	6844	1424	60	standard
L0F-TL1nR	5212	6183	971	6655	ND	970	62	standard

Conserved primers for DMI1-homolog amplification: To identify the symbiosis-affecting mutation in pea line Risnod27, a universal primer set for the PCR amplification of *M. truncatula DMI1* homologs was developed and applied to Risnod27 and its parental cv. Finale. The amplification primers designed from the conserved stretches of *M. truncatula DMI1* genomic sequence are present in Table 1. Denotation of primers follows the alphabetical marking of *MtDMI1* exons 1 - 12 as A - L. Three amplification primers of 20 nt in length, located in the intron regions, were derived from *P. sativum* sequence as a result of primer walking (marked with Ps).

Ten primer pairs provided nine overlapping fragments (Table 2), one of them being prepared in a two-phase TAIL-PCR that covered the entire *DMI1*-homologous region. The PCR fragments were sequenced using the amplification primers (Table 1), and additional sequencing primers were generated by primer walking. The overlap of PCR fragments confirmed the presence of a single *DMI1* homolog in the *P. sativum* genome. The *DMI1*-homologous sequence of a total length of 7 241 bp was obtained for two pea cultivars, Finale and Bohatýr (accession no. AJ973194). No polymorphism was revealed even in the putative non-coding regions, as had been previously reported (Novák *et al.* 2007).

The terminal primers A3F and L1R were also successfully used for reverse transcription (from L1R) and subsequent PCR amplification of most of the SYM8 cDNA.

Identification of the Risnod27 mutation indicates SYM8/DMI1 orthology: Upon identifying the mutation in the asymbiotic pea line Risnod27, its linkage to the mutant phenotype was demonstrated, showing that the identity of the amplified *DMI1* homolog was *SYM8*.

To detect the mutation, equimolar mixtures of PCR fragments (Table 2) amplified from Finale and Risnod27 DNA were subjected to denaturation, hybrid reannealing, and treatment with the nuclease S mismatch-specific endonuclease. The presence of new fragments in the treated C2F-G1R amplification product initiated the sequencing of this fragment in the mutant line Risnod27, confirming the presence of a C→T transition in position 1676 from the translation start of the genomic sequence. This single nucleotide change was absent from other pea genotypes, namely Risnod25, R25 and the distant breeding line of field pea, Zhodino E900.

An allele-specific PCR reaction of the ARMS (amplification refractory mutation system) type was developed to track the presence of the Risnod27 point mutation in plant crosses. The forward primers WtRN27F (5‘-CTTATGTAGCCGACGCAGGAAATC-3’) and Mut RN27F (5’-CTTATGTAGCCGACGCAGGAAATT-3’), as well as reverse primers WtRN27R (5’-CCCATTCCT TCCGTTTCAGCATG-3’) and MutRN27R (5’-CCCATT CCTTCCGTTTCAGCATA-3’), were designed to correspond to the C→T transition at their 3’-ends, to maximize priming specificity. When combined with primers D2bR (Table 1) and A4bF (5’-TTGGCATTGGCTCCACCT CCAG-3’), the reactions yielded an upstream fragment of 1 499 bp and a downstream fragment of 603 bp. Fragments were easily distinguishable with agarose electrophoresis enabling us to discriminate between the wild-type and mutant alleles. An annealing temperature of 70 °C was applied for all four variants.

Using the validated ARMS assay, the presence of both alleles was tracked in the segregants of the B_6 generation from the recurrent backcrossing scheme aimed at transferring the Nod^- trait from the donor line Risnod27 to the recipient pea cultivar Bohatýr. In the ten tested independent B_6 segregants exhibiting the Nod^- trait, only the mutant allele was detected (Fig. 1). The probability of such result in independent inheritance between the observed nucleotide polymorphism and the non-nodulation trait is 1.1×10^{-8}, taking the backcrossing scheme started from a Nod^- segregant in the F_2 into account.

Consequently, the *P. sativum* homolog of *M. truncatula* gene *DMI1* was shown to be the symbiotic gene *SYM8* (Kneen *et al.* 1994) by the exclusive presence of a single nucleotide substitution in the asymbiotic mutant line Risnod27 (sym8) and by the cosegregation of

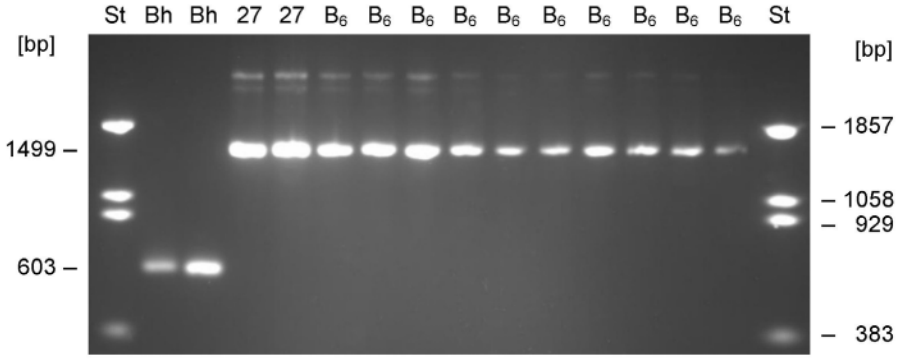

Fig. 1. Cosegregation of a point mutation in pea *DMI1* homolog with asymbiotic phenotype. The light (603 bp) PCR product indicates a positive reaction for the wild-type allele with WtRN27F-D2bR primer pair while the heavy product (1499 bp) of A4bF-MuRN27R indicates the mutant allele present in the line Risnod27. Both reactions were carried out separately and their products were pooled before electrophoresis. *Bh* - cv. Bohatýr (wild-type), *27* - mutant line Risnod27, *B_6* - non-nodulating segregants from the backcrossing of Risnod27 on Bohatýr, *St* - standard ladder (pBR322 cut with MvaI).

the DNA polymorphism with a mutant phenotype. This result is fully consistent with the recently published evidence of *SYM8* - *DMI1* identity based on the localization of five independent sym8 mutations in the *DMI1*-homologous region (Edwards *et al.* 2007). In this way, the earlier assumption made by Schneider *et al.* (2002) and Ané *et al.* (2002) regarding the homology of *DMI1* and *SYM8* has been fully confirmed.

Plant homologs of *DMI1* can be tracked back to *Bryopsida* (Zhu *et al.* 2006, Riely *et al.* 2007), although the elucidation of the origin and evolution of these genes will be facilitated by data from additional species. We hope that the system of conserved primers that we have used will be useful in amplifying and characterizing the homologs from leguminous as well as nonleguminous plants, thus circumventing the necessity of isolating genomic clones.

SYM8 divergence from DMI1: The extent of the *SYM8* coding region and its intron/exon boundaries were inferred from "spliced alignment" (Brendel *et al.* 1998) with the *M. truncatula DMI1* coding sequence (Fig. 2) and confirmed by sequencing the cDNA fragment amplified between the terminal primer binding sites A3F and L1R. Like the other known genomic sequences of the homologous genes of *M. truncatula*, *L. japonicus*, *A. thaliana* and *Oryza sativa*, *SYM8* comprises twelve exons (1 - 12) separated by eleven introns (1 - 11).

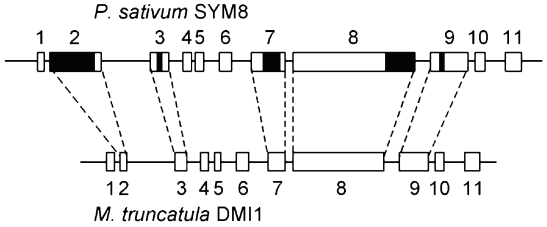

Fig. 2. Position and size of insertions (*solid boxes*) in the introns of *Pisum sativum DMI1* homolog (*SYM8*) compared to *Medicago truncatula DMI1*.

After being compared to the close model species *M. truncatula*, five large insertions can be detected in introns 2, 3, 7, 8 and 9 that extend 558, 70, 218, 362 and 53 bp, respectively. Although no structural similarity was found among the insertions into *SYM8*, some insertions, namely those in introns 2, 7 and 8, are partially homologous to elements occurring in the *M. truncatula* sequenced genome. The direct or inverted repeats that can be detected in these insertions suggest their retrotransposon/transposon origin. The insertion in intron 7 contains terminal inverted repeats of 16 bp (TACTMCCTCCGTTTTTTT) where the first 12 bp correspond to the inverted terminal repeat of *Stowaway* MITE elements, extended by the duplicated target sequence TA flanking the insertion (Bureau and Wessler 1994, Macas *et al.* 2005). On the other hand, the insertion in intron 8 is flanked by the putative target site duplication TTATC and by poorly conserved direct repeats delimited by GT and AC pairs matching the features of a terminal repeat retrotransposon in miniature (TRIM), as described by Witte *et al.* (2001).

The presence and structure of insertions in the pea ortholog is consistent with the role of mobile elements in the expansion of the pea genome during recent evolution, as revealed by comparisons with species of the related genus *Vicia* (Ellis *et al.* 1998, Macas *et al.* 2005, Kovářová *et al*. 2007).

Variable region of DMI1 homologs and its putative significance: The deduced amino acid sequence of the SYM8 protein (894 residues) differs from the *M. truncatula DMI1* product in length only by 12 amino acids, mostly due to a 6-amino acid insertion between Ser192 and Asn199 (corresponding to Asp186-Asn187 of DMI1). Both the *M. truncatula* and *L. japonicus* homologs are thought to code for ion channels, as inferred by their similarity to the Ca^{2+}-gated K^{+}-channel of *Methanobacterium thermoautotrophicum* (Ané *et al.* 2004, Imaizumi-Anraku *et al.* 2005, Edwards 2007). Typical features of ion channels have been reported around the fourth major hydrophobic domain, namely the selectivity filter, inner helix, and gating hinge (Imaizumi-Anraku *et al.* 2005).

A surprisingly low degree of amino acid conservation is observed at the N-terminus of DMI1 homologs up to SYM8 residue Asn238 (Glu226 of *M. truncatula* DMI1), corresponding to a position in the middle of exon 3. The conservation between DMI1 and SYM8 is generally 88.2 %, while the conservation of amino acids N-terminal to Ser239 is only 60.5 %, in contrast to the 98.2 % identity C-terminal to this position. Within the moderately variable region, three extremely variable parts can be distinguished after SYM8/DMI1/POLLUX alignment (Fig. 3), spanning from Pro8 to Thr14, from Ala26 to Ser51 and from Asn86 to approximately Gln125 of SYM8, which correspond to Ser8-Met14, Pro26-Thr50 and Lys84-Lys120 of *M. truncatula* DMI1, respectively.

The hydrophobicity profile of SYM8 according to Cornette *et al.* (1987), partially presented in Fig. 4, confirmed the presence of four major hydrophobic domains as putative transmembrane regions (Ané *et al.* 2004, Imaizumi-Anraku *et al.* 2005). In addition, three minor peaks of hydrophobicity are seen close to the N-terminus with maxima at amino acids 36, 66 and 120. In the alignment of *P. sativum* and *M. truncatula*, the first minor hydrophobic peak coincides with the highly variable region Ala26-Ser51, while two of the minor hydrophilic domains correspond to the highly variable regions Pro8-Thr14 and Asn86-Gln125 (Fig. 4).

The N-terminus of the deduced SYM8 protein also contains a chloroplast signal peptide of 34 amino acids for plastid localization, as determined by B. Riely (personal communication). The length of the signal peptide was delimited by *ChloroP* prediction software.

The low degree of conservation at the 5'-end of *DMI1* homologs (Fig. 3) might be crucial for understanding the function of their products. In light of the view that the

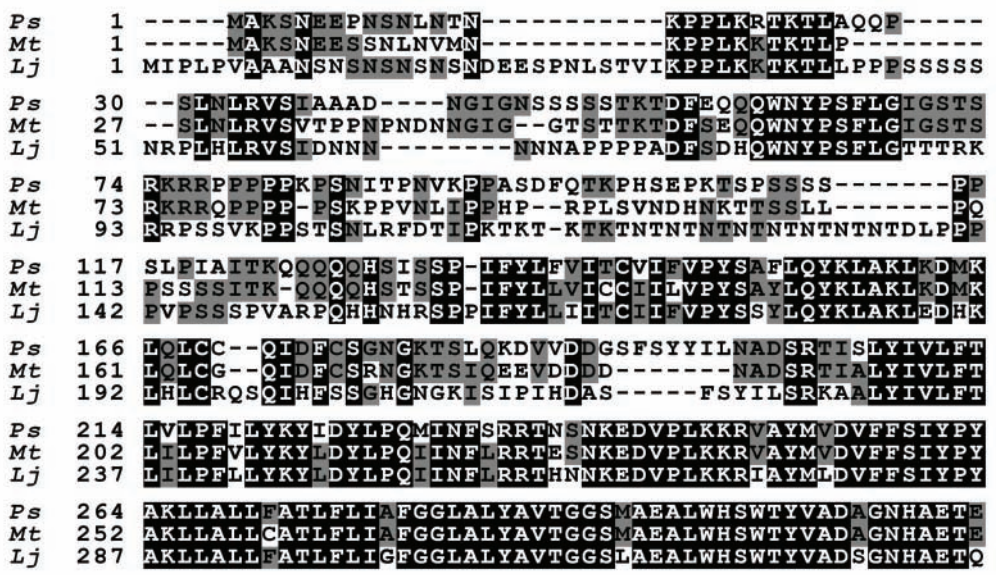

Fig. 3. Highly variable regions in the N-proximal part of *Pisum sativum SYM8* (denoted as *Ps*) predicted product as delimited in the multiple alignment with *Medicago truncatula* DMI1 (*Mt*) and *Lotus japonicus* POLLUX (*Lj*) proteins. Fully conserved amino acids are on *black background*, partially conserved are *shaded grey* while the non-conserved positions are *not shaded*.

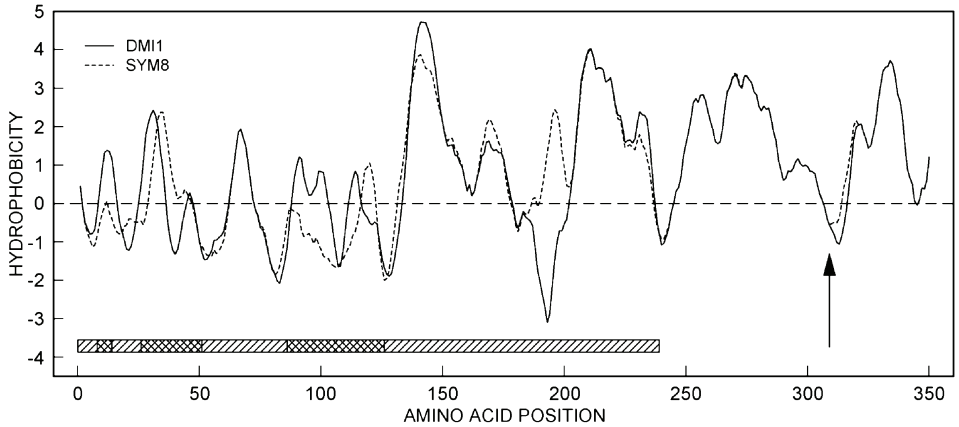

Fig. 4. Location of variable regions relative to the hydrophobic domains in the N-proximal part of SYM8. The mean hydrophobicity profiles were built using Cornette scale, scan window size was 11 and linear reduction of weights ranged from 100 % (center) to 0 (edges). The gaps in the aligned DMI1 were not abridged. The moderately variable regions are indicated with *hatched boxes* while the hypervariable regions are *crosshatched*. The variable region borders were derived from the alignment *Pisum sativum* SYM8/*Medicago truncatula* DMI1/*Lotus japonicus* POLLUX. *Arrow* indicates the position of a mutation in line Risnod27.

rhizobial Nod factor has a variable structure as a prerequisite of its activity in different legume groups (Spaink 2000), it is tempting to speculate that the variable portion of DMI1 homologs also takes part in the recognition of these molecules. This function could be accomplished in parallel to LysM-type receptor kinases where several independent kinds of evidence exist for their role as Nod factor receptors (Limpens *et al.* 2003, Radutoiu *et al.* 2003, 2007). It is conceivable that the Nod factor molecule might serve as a ligand gating the channel function of DMI1 homologs. The finding that the N-proximal part of *M. truncatula* DMI1 is indispensable for plant symbiotic responses, even when its plastid targeting function has been compensated with a heterologous sequence (Riely *et al.* 2007), represents circumstantial evidence for multiple roles of this part of the molecule. On the other hand, the highly conserved C-terminal portion of the SYM8 protein might be involved in the structurally constraining interaction with down-stream members of the signal transduction chain.

The final orientation of the SYM8 molecule in the target membrane is of key importance for understanding its function. Notably, *M. truncatula* DMI1 has recently been shown to be concentrated in the nuclear membrane (Riely *et al.* 2007), although the prediction algorithms point to the plastid compartment, consistent with the

L. japonicus homolog POLLUX (Imaizumi-Anraku *et al.* 2005). This disparity and different length of the predicted SYM8 signal peptide (n = 34) compared to *M. truncatula DMI1* (n = 69) might reflect a different pattern of organelle targeting in pea. It could still be possible that a minor fraction of SYM8 molecules are integrated into proplastids abreast with the nucleus. In this case, the plastid signal sequence would be responsible for transport into the proplastid stroma and, after cleaving the first stretch, for further sorting into the inner plastid envelope (Li and Schnell 2006). The resulting four-pass molecule should expose both ends to the stroma. Nevertheless, outer exposure of the variable region might be enabled by an additional membrane span due to the short hydrophobic domains at the N-end (Fig. 4).

Further experimental evidence is necessary to completely exclude alternatives in the intracellular localization of SYM8 and to establish its transmembrane orientation, which can be different from predictions based on sequence data.

The Risnod27 mutation directly affects ion channel function: The point mutation in the Risnod27 line is in position 925 of the putative coding sequence, corresponding to the conserved part of exon 3. The caused single amino acid change of histidine 309 to tyrosine (corresponding to His297 of *MtDMI1*) is located in the hydrophobicity minimum between the third and fourth transmembrane domains (Fig. 4); however, the mutation itself was accompanied by a negligible change in local hydrophobicity (not shown). The Risnod27 mutation changed the sixth of seven amino acids forming the selectivity filter region of the predicted ion channel (Imaizumi-Anraku *et al.* 2005).

The primary structure of the MtDMI1 selectivity filter in pea SYM8 is AlaAspAlaGlyAsnHisAla, differing from the canonical plant structure found in the *O. sativa* and *L. japonicus* homologs only in that it has Ala instead of Ser in the third position. On the other hand, the *A. thaliana* homolog differs from the canonical selectivity filter by having serine in the fifth position. The primary structure of the selectivity filter in plant homologs substantially differs from archaebacteria and was treated as a sign of specificity for another ion (Ané *et al.* 2004, Imaizumi-Anraku *et al.* 2005). Compared to the selectivity filter of the archaebacterial molecule (ThrValGlyTyrGlyAspTyr), the basic amino acids histidine in position 6 and asparagine in position 5 of the legume sequences are notable and, as evidenced by Risnod27, at least histidine is essential for symbiotic function. The other basic amino acid, asparagine adjacent to histidine 309, is missing only in the representative of an asymbiotic crucifer family.

The function of histidine 309 is thought to be associated with dehydration of transported potassium cations (Edwards *et al.* 2007), according to the model accepted for animal channels (Morais-Cabral *et al.* 2001). The function of the plant homologs as potassium channels has recently been supported by the direct monitoring of channel function in reconstituted lipidic membranes (Charpentier *et al.* 2007). The assumed function of His309 in ion dehydration is also consistent with our finding that the Risnod27 phenotype can be partially recovered by water stress.

On the other hand, the basic character of the key amino-acids in the selectivity filter of DMI1 homologs still allows for speculation about other ionic interactions. Oxyanions with a negatively charged oxygen matching the histidine residue are the most likely candidates, *e.g.*, bicarbonate, nitrate and dihydrogenphosphate. Additional interaction with the amide group of asparagine neighboring histidine 309 can be mediated by a hydrogen-bond forming oxygen of the oxyanion. Taking into account the similarity of the ionic filter motif to the anion-binding cavities of the nitrate- and bicarbonate-transporting and binding proteins NtrA, NtrC, CmpA and CmpC of *Synechocystis* chloroplasts (Koropatkin *et al.* 2006), nitrate is the most fitting anion. Notably, the presence of histidine among the anion-interacting amino acids in *Synechocystis* proteins is essential for nitrate selectivity.

Our data shows that Risnod27 differs from other sym8 mutants described (Kolycheva *et al.* 1993, Albrecht *et al.* 1998) in its ability to form endomycorrhizal symbiosis. This observation suggests that the residual activity of the channel is sufficient for mycorrhiza development while other functions performed by the molecule and affected by mutations in the alleles of Risnod25 and R25 (Edwards *et al.* 2007) are indispensable for both types of symbioses. Surprisingly, the non-symbiotic traits of Risnod27 are affected in no degree by the symbiotic mutation, as shown in studies using Risnod27 as a reference line for symbiotic nitrogen fixation assays (Škrdleta *et al.* 1995, Biedermannová *et al.* 2002). The pleiotropic effects of SYM8 channel inactivation, if present, might be useful in determining the original function of DMI1 homologs before their recruitment for symbiosis establishment.

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