

Subcloning of Nodulin 26 Wild Type(S262) and Phosphorylation Site Mutant(S262D) into the Yeast Expression Vector pYES2

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Abstract

Wild type nodulin 26(nod 26) cDNA(S262) and phosphorylation site mutant(S262D) were constructed by a yeast expression system using pYES2. Cloned pYES2 plasmids(pYES2-S262 and pYES2-S262D) were screened by restriction mapping with *Bam*HI or *Kpn*I. S262 nod 26 contained a serine residue at position 262 and S262D nod 26 contained the substitution mutation of serine to aspartic acid residue at position 262 were verified by automated fluorescent DNA sequencing.

Key words: nodulin 26, plasmid, restriction enzyme, sequencing

INTRODUCTION

Glycine max(soybean) is of the Leguminosae family of plants characterized by the ability to establish a nitrogen fixing symbioses with soil bacterium of the genus *Bradyrhizobium*(1). *Bradyrhizobium japonicum* are responsible for nitrogen fixation in soybean and are found within a specialized root organ termed the nodule(2).

Two types of cells are found within the nodule: infected and uninfected cells. Infected cells are a specialized organelle, encapsulating the bacteroids, termed the symbiosome(3). The symbiosome membrane is a semi-permeable barrier that mediates communication and metabolic transport(4). In particular, this membrane allows for the export and assimilation of fixed nitrogen.

Several nodule-specific proteins are expressed during the formation of the nodule to aid in the establishment and maintenance of the symbiosis. Nodulin 26(nod 26) is a major integral transmembrane protein, as a member of major intrinsic protein family(5,6) found within the symbiosome membrane(7,8). This family contains more than 50 members which share significant amino acid sequence identity(30~40%), have similar putative membrane topologies(six transmembrane domains with hydrophilic amino and carboxy terminal domains oriented towards the cytosol(Fig. 1), and have diverse transport capabilities including water, solute and ion channel activities(9).

Roberts and coworkers(10,11) are interested in nod 26's role in nodule physiology. Nod 26 is phosphorylated on Ser262 by a calmodulin-like domain protein

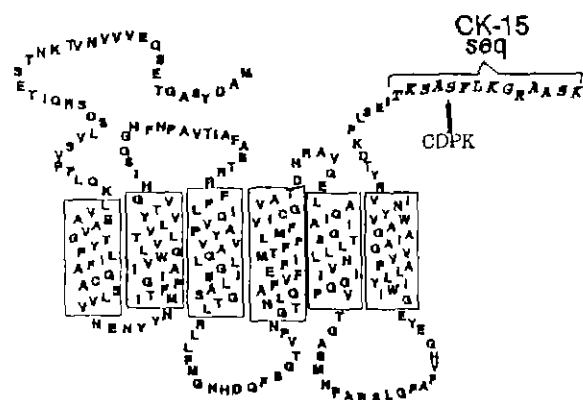


Fig. 1. Sequence and proposed topology of nodulin 26 (2).

The CK-15 sequence which contains the phosphorylation site(Serine 262) and recognition sequence for CDPK is indicated.

kinase. Upon reconstitution, into planar lipid bilayers, nod 26 demonstrated ion channel activity with a maximal single channel conductance of 3.1 nS and upon phosphorylation on Ser262, nod 26 showed a voltage dependent gating activity(12).

To further investigate nod 26's permeabilities, as well as the role of phosphorylation on these activities, nod 26 wild type(S262) and phosphorylation site mutant(S262D) cDNAs were cloned onto the yeast expression vector pYES2.

MATERIALS AND METHODS

Materials

The JM101 glycerol stocks containing pYES2 plasmid

were a gift of Dr. Jeffery M. Becker (Dept. of Microbiology, University of Tennessee, USA). The JM101 glycerol stocks containing pRSETA with S262 or S262D nod 26 genes were a gift of Dr. Daniel M. Roberts (Dept. of Biochemistry, Cellular and Molecular Biology, University of Tennessee, USA). *Bam*HI, DNA ligase and Wizard plus DNA purification system were purchased from Promega corporation (Madison, WI, USA). Alkaline phosphatase and *Kpn*I were obtained from Amersham corporation (Arlingto Heights, IL, USA). pYES2 was purchased from Invitrogen corporation (Carlsbad, CA, USA). Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Subcloning to yeast expressing vector

Yeast expression vector pYES2 containing S262 or S262D nod 26 gene was constructed by a expression cloning strategy as shown Fig. 2. Plasmid pYES2 into

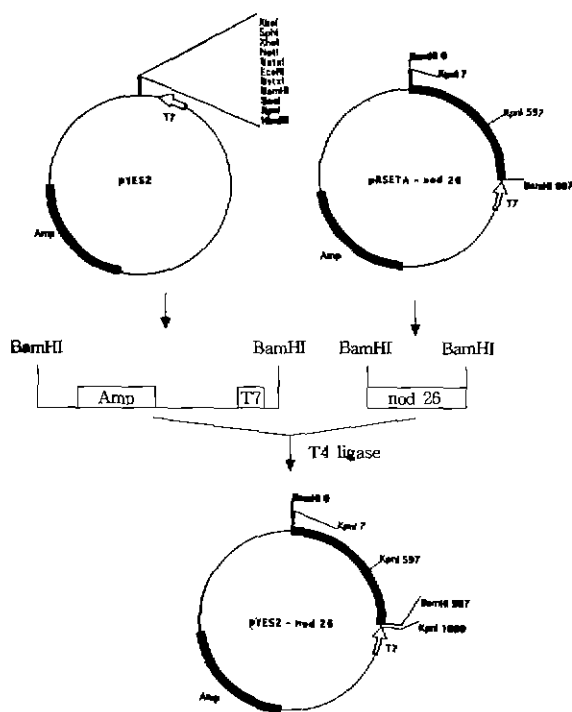


Fig. 2. Cloning of nodulin 26 gene into yeast expression plasmid pYES2.

S262 or S262D nodulin 26 gene fragment (*Bam*HI fragment) derived from cloned pRSETA-nodulin 26 is inserted into *Bam*HI cleaved pYES2 vector by treatment with T4 DNA ligase as described in the Materials and Methods. Indicated on the plasmid are the nodulin 26 gene (nod 26), bacteriophage T7 promoter (T7), ampicillin resistance gene (Amp).

Table 1. Ligation reaction procedures¹⁾

No. of Tube	Sample	Insert S262	Insert S262D	pYES2	H ₂ O	2×DNA ligase
1	pYES2+S262	5μl	-	2.2μl	2.8	10μl
2	pYES2+S262D	-	3.6μl	"	4.2	"
3	pYES2	-	-	"	7.8	"
4	S262	5μl	-	"	5	"
5	S262D	-	3.6μl	"	6.4	"

¹⁾ Added pYES2 vector and H₂O into melted insert. After equilibration by placing 37°C for 10min, then 2×DNA ligase was added and incubated 16°C overnight.

JM101 was minipreped and digested with *Bam*HI. Alkaline phosphatase was then added to the digest and the mixture was incubated. Plasmid pRSETA(13) containing the nod 26 cDNA, S262 or S262D, were digested with *Bam*HI. Digested DNA fragments were separated by 0.8%(w/v) low melting point(LMP) agarose gel electrophoresis and extracted from the sliced gel by using a modified protocol(14) from the method developed by Maxam and Gilbert(15). The nod 26 gene fragments and the *Bam*HI-cleaved pYES2 vector were ligated to generate pYES2-nod 26(16). Isolation S262 and S262D nod cDNAs were inserted into the *Bam*HI site of the *Bam*HI cut pYES2 vector by use of the enzyme DNA ligase. As shown in Table 1(Tube No. 1 and 2), the nod 26 cDNA's were added to the linearized pYES2 vector, DNA ligase was added and the mixture was incubated overnight at 16°C. Negative control reaction mixtures were also setup(Tube No. 3-5). Plasmid pYES2-nod 26 was transformed into *E. coli* JM101. Isolation and purification of plasmid DNA were carried out by the alkaline lysis method of Birnboim and Doly(17). Restriction endonuclease, T4 ligase, and alkaline phosphatase were used under conditions specified by commercial supplier.

Bacteria culture

E. coli cell cultures were grown overnight at 37°C with shaking and centrifuged at 1,400×g at 4°C for 10min. The pellet was washed by resuspending solution of 50mM Tris-HCl, pH 7.5 containing 10mM EDTA and 100μg/ml RNase A. Then, the samples were centrifuged at above conditions. The pellet was resuspended with 300μl of 50mM Tris-HCl(pH 7.5) buffer containing 5mM EDTA and 2mM β-mercaptoethanol. The cells were stored at -80°C.

RESULTS AND DISCUSSION

Preparation of pYES2 vector and nod 26 cDNA

The isolated pYES2 vector was linearized by digestion at 37°C for 2hrs in a reaction mixture containing *Bam*HI and alkaline phosphatase as shown(A) in Fig. 3. The nod 26(S262) and phosphorylation site mutant(S262D) cDNA's were excised from the *Bam*HI site of the pRSETA vector by digestion with *Bam*HI (B) in Fig. 3. S262 and S262D nod 26 cDNA fragments

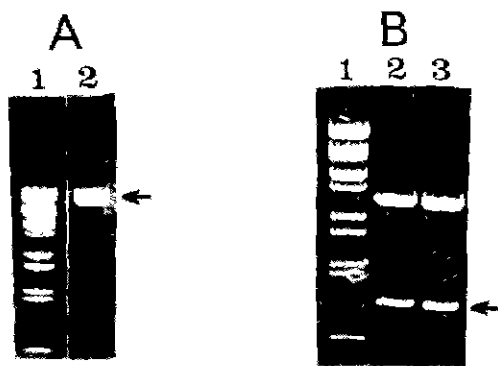


Fig. 3. Agarose gel electrophoresis of digested pYES2 vector(A) and pRSETA with nodulin 26 cDNAs (B) by *Bam*HI.

(A) pYES2 vector was linearized by alkaline phosphatase treatment after *Bam*HI digestion; lane 1, marker DNA; lane 2, linearized pYES2(5.7kb). (B) DNA fragments(987 bp) of S262(lane 2) and S262D (lane 3) from pRSETA containing nodulin 26 gene.

(987 bp) were resolved from vector DNA by 0.8%(w/v) LMP agarose gel electrophoresis. The nod 26 cDNA band was isolated from the LMP gel using a razor blade.

Transformation

As shown in Table 2, competent JM101 *E. coli* were transformed with each of the 5 ligation reactions shown in Table 1. In addition, cells were transformed with uncut pRSETA vector as a positive control to ensure the competency of the JM101 cells. To test cell viability and ampicillin selection, untransformed cells were plated.

pYES2 contains an ampicillin resistance marker, therefore only cells containing religated pYES2 and cells with nod 26 inserted into pYES2 will be viable on ampicillin plates. Plate numbers 5 and 6 on Table 2, represent cells transformed with ligated pYES2 alone. In this control no colonies are expected. The appearance of colonies indicates the alkaline phosphatase treatment was incomplete. Therefore colonies in plate number 1 ~ 4 will contain either all religated vector or mixture of religated vector and vector with nod 26 inserted. Further screening is necessary to distinguish which colonies on plate number 1 and 3 plates have the nod 26 gene inserted into the pYES2 vector.

Screening

As shown in Fig. 4, nod 26 can be inserted into the

Table 2. Transformation in JM101 procedures¹⁾

Sample	Number of cell	Volume of ligation mix	DNA	Volume of cell plated	Type of plate	Number of colony	#plate
pYES2+S262	1	5 μ l	50ng	100 μ l	LB/AMP	328	1
				900 μ l	"	684	2
pYES2+S262D	2	"	"	100 μ l	"	280	3
				900 μ l	"	484	4
pYES2	3	"	8ng	100 μ l	"	105	5
				900 μ l	"	352	6
S262	4	"	12.5ng	100 μ l	"	0	7
				900 μ l	"	0	8
S262D	5	"	12.5ng	100 μ l	"	0	9
				900 μ l	"	0	10
Uncut pRSETA	6	"	5ng	100 μ l	"	many	11
				900 μ l	"	many	12
No DNA	7	"	0ng	450 μ l	"	0	13
				450 μ l	LB	Lawn	14

¹⁾Ligated sample was added into JM101 competent cells and incubate on ice for 30 min, then heat shock process was done at 42°C for 90 seconds. After adding 900 μ l of Luria Borth media and incubate at 37°C for 2 hrs with gently shaking and plated out the cells as shown in reference 16. LB, Luria broth agar; Amp, Ampicillin

pYES2 vector in one of two possible orientations: sense or antisense. Colonies were screened to determine if the pYES2 vector contained the nod 26 gene and in what orientation the nod 26 gene was inserted. The screening process first involves isolation of plasmid DNA from colonies and then restriction enzyme digestion of the DNA.

As illustrated in Fig. 5, *Bam*HI digestion will indicate the presence of the nod 26 gene(987 bp) but gives no information as to the orientation of the nod 26 cDNA. *Kpn*I, however, will give a different banding pattern if the orientation is sense(2 bands: 390 and 590) verse antisense(2 bands: 7 and 590). In our study, a sense insertion will give 2 bands upon digestion with *Kpn*I whereas an antisense insertion will give only one band visible by 1.2% agarose gel electrophoresis(a second smaller will also result but it is too small to see on the gel) as shown in Fig. 5. Fig. 5 depicts a 1.2% agarose gel representing sense orientation [lanes 12(S262) and 17 (S262D)] and antisense orientation(lanes 13, 14, 15, 16, 18, 19, 20, 21).

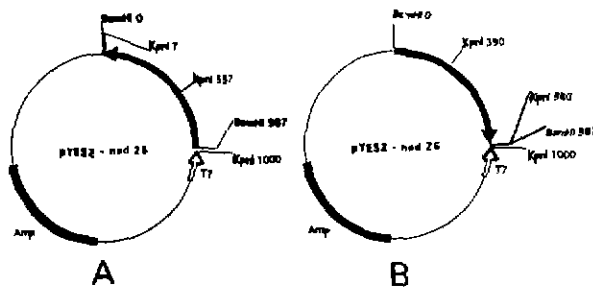


Fig. 4. Illustration of cloned pYES2-noduline 26 gene by sense(A) verse antisense(B) orientation. Indicated on the plasmid are the nodulin 26 gene(nod 26), bacteriophage T7 promoter(T7), ampicillin resistance gene(Amp).



Fig. 5. Agarose gel electrophoresis of screening digestion. Cloned pYES2 plasmids(pYES-S262 or pYES2-S262D) were screened by restriction mapping with *Bam*HI or *Kpn*I. Lane 1, marker; lane 2~6, digested S262 by *Bam*HI; lane 7~11, digested S262D by *Bam*HI; lane 12~16, digested S262 by *Kpn*I; lane 17~21, digested S262D by *Kpn*I.

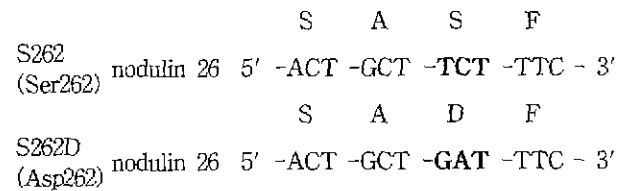


Fig. 6. DNA sequence of S262 and S262D nodulin 26 cDNAs around the phosphorylation site.

Sense colonies of both S262 and S262D nod 26 were stored as glycerol stocks(-80°C) for the future use.

Sequencing

Automated fluorescent DNA sequencing was done to verify that S262 nod26 contained a serine residue at position 262 and that S262D nod26 contained an aspartic acid residue at position 262. Fig. 6 shows the DNA sequence around the phosphorylation site.

Future studies

The isolated newly cloned plasmids(pYES2-S262 and pYES2-S262D nod 26) are currently being transformed into a yeast sec-strain. Once expression of nod 26 is established, the yeast secretory vesicles(loaded with nod 26 protein) will be isolated and used to study water and solute permeabilities of nod 26. The phosphorylation site mutant nod 26(S262D) will be used to elucidate the role of phosphorylation on nod 26's permeabilities.

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(Received February 11, 1997)