Expression of *Lupinus luteus* nodulin-45 gene in *E. coli* cells. Mutagenesis of coding sequence by PCR method

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The nodule specific cDNA clone — pLN-13 [1] from yellow lupin encodes a single polypeptide of molecular mass 45 kDa (called nodulin-45; N-45). Figure 1 shows a full length cDNA copy of pLN-13. A deduced amino-acid sequence reveals that this protein is highly hydrophilic with putative N-terminal signal peptide and two asparagine-linked glycosylation sites. The N-45 cDNA clone (1399 bp) represents one of the abundant sequences of lupin cDNA library. A coding region of 1200 bp is located between nucleotides #41 and #1240 of the full copy of pLN-13 cDNA clone (subcloned into pBluescript SK at PsiI of multiple cloning site). The function and subcellular localization of nodulin-45 still remains unclear.

To prepare N-45 protein for functional studies and determination of subcellular localization we decided to express its cDNA clone in *E. coli* cells in the pET-3a expression vector [2]. In this system, synthesis of large amounts of foreign gene products is directed by phage T7 gene 10 promoter, which uses T7 RNA polymerase. It is well known that the polymerase transcribes the gene 10 promoter very efficiently.

The nodulin-45 cDNA coding fragment of 1200 bp has been amplified by PCR. As a target for the amplification reaction, a full cDNA copy (1399 bp) inserted to pBluescript SK at PsiI site was used. The oligodeoxynucleotide primers (N & C) were synthesized to amplify coding fragment of N-45 cDNA clone in reaction with Taq DNA polymerase. The N-primer containing the sequence recognized by Ndel matches 3'-end of n45 cDNA clone. The C-primer contains the sequence recognized by BamHI and two stop codons. It matches the complementary strand of 5'-end of this clone.

Since BamHI was used for insertion of the amplified sequence into the pET-3a expression vector it was necessary to introduce mutation at the internal BamHI site of cDNA clone. Two additional (internal primers: a and b, Fig. 2) which matched the internal BamHI sequence were mutated at the position corresponding to nucleotide #385 of the n45 clone (G385 → T385). This mutation (in the triplet encoding arginine: AGG → AGT) did not change the amino acid encoded by new triplet (AGT).

The n45 cDNA coding sequence with mutated nucleotide at the position #385 was prepared by two independent ways (variant 1 & 2).

1. Amplification of n45 coding sequence (1200 bp) from pBluescript SK (CC form) carrying a full copy of cDNA clone (pBluescript/n45) using two pairs of primers: external (N & C) and internal (a & b). The molar ratio of target DNA to external and internal primers was 1:10000:10, respectively. PCR products and their restriction fragments after digestion

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Abbreviations: bp, base pair; N-45, yellow lupin nodulin-45; n45, cDNA coding fragment for nodulin 45; nt, nucleotide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium salt of dodecyl sulphate.
Fig. 1. The nucleotide sequence of full copy cDNA clone of pl.N-13.
This is a corrected sequence of Pl.N-13 published earlier [11] which was send to GenBank and registered under the accession No.: X77044.
with BanHI were analyzed by 1% agarose gel electrophoresis [3] (Fig. 3, lines 2, 3). Two PCR products of 1200 bp — with and without internal mutation were observed. The product without internal mutation digested with BanHI yielded two fragments: 400 and 800 bp.

2. Amplification of n45 coding sequence (1200 bp) from the linearized form of pBlue-script/n45 (with BanHI). This approach was carried out to increase the yield of the product with internal mutation. PCR products and their restriction fragments after digestion with BanHI were analyzed on agarose gel (Fig. 3, lines 5, 6).

Fig. 3. Restriction analysis of cDNA coding fragment of N-45.
1. pBlue-script/n45 digested with PstI: 2964 bp — linearized pBlue-script SK, 1399 bp — full copy of cDNA clone of N-45; 2. products of PCR amplification (1200 bp) with two pairs of primers, var. 1, 3, BanHI restriction fragments of PCR products, var. 1: 1200 bp — cDNA coding fragment of N-45 with mutation at 385 nt, 800, 400 bp — BanHI restriction fragments of amplified cDNA without mutation; 4, pET-3a/n45 digested with BanHI and NdeI: 4640 bp — pET-3a, 1200 bp — n45; 5. products of PCR amplification (1200 bp) with two pairs of primers, var. 2, 6, BanHI restriction fragments of PCR products, var. 2: 1200 bp — cDNA coding fragment of N-45 with mutation at 385 nt, 800, 400 bp — BanHI restriction fragments of amplified cDNA without mutation; 7. pET-3a/n45 digested with BanHI and NdeI: 4640 bp — pET-3a, 1200 bp — n45; 8. pET-3a itself, linearized (with NdeI or BanHI); (cd. 4 & 8: pET-3a/n45 was amplified in E. coli cells, strain HMS174 for isolation of pure plasmid for sequence analysis).
Products of PCR amplifications of the n45-coding fragment with mutation at the #385 nt and pET-3a were digested with Ndel and BamHI and then ligated (pET3a/n45, Fig. 4).

E. coli cells, strain HMS174 [2] were transformed with pET-3a/n45 construct. Growing plasmid was purified on Qiagen column for its nucleotide sequence analysis to determine the right clone (sequence corresponding to the cDNA coding fragment mutated at the position #385 nt). The expression of pET-3a carrying correct n45 coding sequence was performed in E. coli cells, strain BL21(DE3)pLysS with T7 lysozyme activity [2]. T7 RNA polymerase gene was induced by addition to the culture of 0.5 mM IPTG. The products of gene expression were analyzed by 15% PAGE/SDS (Fig. 5) according to Laemmli [4]. The identity between expressed polypeptide and amino-acid sequence of nodulin 45 protein deduced from nucleotide sequence of cDNA clone (Fig. 1) was supported by microsequencing analysis of 12-

N-terminal amino-acid residues of recombinant protein (electroblotted into PVDF membrane).

The applied PCR amplification method resulted in obtaining the designed point mutations within the target sequence when the pair of internal primers was used together with external primers. This method can be used to generate any point mutation at the level of nucleotides and amino acids. Genetically engineered proteins obtained in expression system in bacterial cells can be further studied either by X-ray crystallography or functional assays.

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REFERENCES