

*Communication*

**Conservation of the structure and organization of lupin mitochondrial *nad3* and *rps12* genes<sup>\*⊙</sup>**

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**A high level of the nucleotide sequence conservation of mitochondrial *nad3* and *rps12* genes was found in four lupin species. The only differences concern three nucleotides in the *Lupinus albus rps12* gene and three nucleotides insertion in the *L. mutabilis* spacer. Northern blot analysis as well as RT-PCR confirmed cotranscription of the *L. luteus* genes because the transcripts detected were long enough.**

Plant mitochondrial genomes differ from those of mammals and fungi in respect of size, organization and complexity. The latter seems to be due to homologous recombination between repeated sequences and insertion of chloroplast and nuclear DNA [1]. These processes may account for the variation and a novel species-specific location of genes. To date almost all the genes of protein complexes

in the respiratory chain have been identified in plant mitochondria, among them genes coding for several subunits of complex I. Genes corresponding to subunits *nad1-nad7* and *nad9* were found to be located in the mitochondrial genome [2] but other genes in the nuclear genome. The plant mitochondrial genome also encodes a certain number of ribosomal proteins (above 10) which are scattered

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around the respective genomes. In several instances the ribosomal proteins appear to be linked to the other genes, for example to some genes which encode subunits of complex I. This mechanism was established in the course of seed plant evolution [3].

Previously we analysed the arrangement of the rRNA gene unit and tRNA-like repeat in the mitochondrial genome of three lupin species, and found some differences between them [4]. To obtain further information about the organization and conservation of other genes in those lupin species we have started to characterize some subunits of the complex I dehydrogenase. The data about subunits of plant mitochondrial dehydrogenases are very scarce, especially in the family *Fabaceae*.

The aim of this study was to analyse the structure and arrangement of one of those subunits, named *nad3* and linked with *rps12*, as well as their expression.

## MATERIALS AND METHODS

**Plant material.** Seeds of *Lupinus luteus* L. cv. Topaz, *L. angustifolius* L. cv. Mirela and *L. mutabilis* L. pop. 21757 were from the Plant Breeding Station Wiatrowo (Poland). Seeds of *L. albus* L. cv. Hetman were obtained from the Plant Breeding Station Kosieczyn (Poland).

**DNA preparation.** Mitochondrial DNAs of 6-day-old etiolated seedlings of *Lupinus* species were prepared as described by Karpińska & Augustyniak [5]. Total DNA of 3-day-old etiolated seedlings of *L. mutabilis* was prepared as described by Zimmer & Newton [6] with the following modifications: the material was ground in the presence of liquid N<sub>2</sub>, and DNA was dissolved at room temperature instead of 65°C.

**Preparation of mitochondrial RNA.** Mitochondria for isolation of RNA were not treated with deoxyribonuclease I. RNA was prepared as described by Chomczyński & Sacchi [7] with additional 2 M LiCl precipitation.

Mitochondrial RNA was also treated with deoxyribonuclease free RNases (Promega).

**PCR amplification of DNA.** The PCR reaction mixture contained in 20 µl the following components: 50 ng of mitochondrial or total DNA, 0.5 µM of each primer, 10 mM Tris/HCl, pH 8.8, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% (v/v) Triton X-100, 200 µM of each dNTP and 0.03 units of Prime Zyme DNA polymerase (Polygen, Wrocław, Poland) per microlitre of the reaction mixture. Two synthetic primers P<sub>1</sub> and P<sub>2</sub> specific to the wheat DNA fragment including the *nad3* and *rps12* genes were used. PCR was carried out in a Biometra cycler. For the above primers one-step amplification was used: the initial denaturation at 95°C for 5 min was followed by 30 cycles of: 1 min at 95°C, 1 min at 55°C and 2 min at 72°C. The PCR was terminated by an elongation cycle of 10 min at 72°C. PCR products were analysed on 1% agarose (Promega) gels. The expected fragments were recovered from agarose and purified on glass wool or on Microcon S-100 microconcentrators (Amicon), and PCR reamplified.

**Sequencing of PCR fragments.** Sequencing of DNA was conducted using "f-mol Kit" (Promega). For the sequencing of PCR fragments four additional primers, P<sub>3</sub>, P<sub>4</sub>, P<sub>5</sub>, P<sub>6</sub> were used. The whole region containing *nad3* and *rps12* genes was sequenced on both strands twice, sometimes even three times. Nucleotide sequence analysis was performed with the DNASIS program. The homology values were obtained including regions corresponding to primers P<sub>1</sub> and P<sub>2</sub>.

**Labelling of DNA.** RNA was probed with the DNA fragment containing both the *nad3* and the *rps12* gene, as well as fragments containing the *nad3* or the *rps12* gene alone. All specific probes were purified on Microcon S-100 and <sup>32</sup>P-radiolabelled using random hexamer primers [8].

**Northern blot analysis.** Samples of *L. luteus* mitochondrial RNA (13 µg) were fractionated on 1.5% denaturing formaldehyde agarose gels and transferred directly to a nylon

membrane Hybond N (Amersham) in  $20 \times$  SSC. Hybridization was carried out at  $42^\circ\text{C}$  for 18 h in a mixture of 50% formamide,  $10 \times$  Denhardt solution, 50 mM Tris/HCl, pH 7.5, 0.1% SDS and  $100 \mu\text{g/ml}$  salmon sperm DNA. Prehybridization was performed in the same solution which contained additionally 1 M NaCl. The membrane was washed twice in  $2 \times$  SSC at room temperature for 5 min, twice in  $2 \times$  SSC, 0.5% SDS at  $65^\circ\text{C}$  for 15 min and twice in  $0.1 \times$  SSC at room temperature for 10 min.

**Reverse transcription of RNA.** The cDNA for 2 separate RT-PCR runs were synthesized from *L. luteus* mitochondrial RNA and specific primers P<sub>2</sub> or P<sub>7</sub> by AMV reverse transcriptase (Promega) following the Promega protocol. Six micrograms of mitochondrial RNA,  $0.5 \mu\text{g}$  of each primer per microgram of RNA and 15 units of AMV reverse transcriptase were used for the reaction. The cDNA synthesis was terminated by extraction with phenol and chloroform. The ethanol precipitated product was dissolved in water and used for amplification and reamplification by PCR.

**List of primers.** Oligonucleotides used for the polymerase chain reaction, sequencing and reverse transcription:

P<sub>1</sub>- 5' ATG TCG GAA TTT GCA CCT ATT-  
TGT ACT 3'

P<sub>2</sub>- 5' TTT CGA TTT AGG TCT TTC TGC-  
ACC 3'

P<sub>3</sub>- 5' GAT TGG ATC TCT CTA TGA ATG-  
G 3'

P<sub>4</sub>- 5' CTC CTC TAC CAT GAC GAA TCA-  
A 3'

P<sub>5</sub>- 5' TGC CCA AGG AAA AGA AAA GGT-  
TA 3'

P<sub>6</sub>- 5' TCA ACG AGA ACA CCG AAA AAA-  
CC 3'

P<sub>7</sub>- 5' TTA CTC CCG ATC CGA AGC ACC 3'

## RESULTS AND DISCUSSION

The mitochondrial and total DNA of the four different lupin species studied were used for amplification of a fragment containing the *nad3* and *rps12* genes. We separated and determined the nucleotide sequence of this fragment in all analysed lupins (EMBL accessions No. AF035355, AF035356, AF035357, AF035358). It turned out that the *nad3* gene had an identical sequence in all four lupin species. The 357 bp-long *nad3* gene showed the highest (95–97%) nucleotide sequence identity with the respective fragment from the mitochondrial DNA of seed plants, 85% identity with liverwort (*Marchantia polymorpha*), and 55–71% identity with *nad3* of algae and fungi.

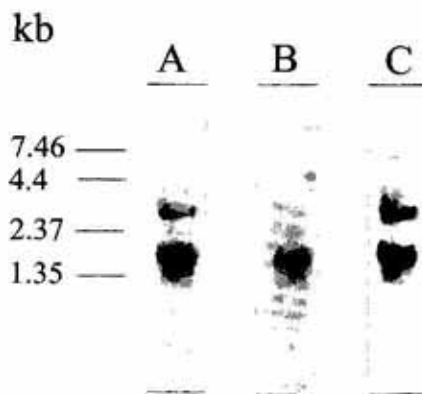
The 378 nucleotide-long *rps12* gene had an identical sequence in three lupin species; a difference in nucleotide sequence was observed only in *L. albus*. The position at the 4<sup>th</sup> and 12<sup>th</sup> nucleotides was occupied by adenosine instead of cytidine present in the other species, and the position 279 by thymine while the rest had guanosine. The lupin *rps12* gene exhibits 81–96% identity with the respective DNA fragment of other seed plants and 79% with liverwort. It is interesting that the mitochondrial *rps12* gene reveals 65–71% identity with the corresponding chloroplast *rps12* gene of higher plants.

A characteristic feature of the spacer region between the *nad3* and *rps12* genes is its variability in length and composition among the different angiosperms [3]. The spacer between the *nad3* and *rps12* genes in three of the analysed lupin species had 48 nucleotides in length, but 51 nucleotides in length in *L. mutabilis*. The additional 3-nucleotide insertion was observed at the position of the 25<sup>th</sup> nucleotide. The lupin spacer represents 70–91% nucleotide sequence identity with other seed plants.

The comparison of the lupin sequence identity of the whole fragment containing the

*nad3* and *rps12* genes revealed 90–96% identity with other seed plants and 80% with liverwort. The results concerning the arrangement and structure conservation agreed well with the data obtained from other angiosperms [3, 9]. One peculiar *nad3* gene containing an additional fragment at 5' part was only found in rice [10]. However, in *Lupinus*, which belongs to one of the oldest genera in evolutionary terms, breeding has not affected the arrangement and nucleotide sequence of the *nad3* and *rps12* mitochondrial genes. The identity in sequence of the *nad3* gene in four lupin species and the high level of conservation of this gene may suggest the importance of this hydrophobic type subunit in electron transfer through the respiratory chain. In this context it would be interesting to test how the editing looks like in the case of *nad3* transcripts in lupin species.

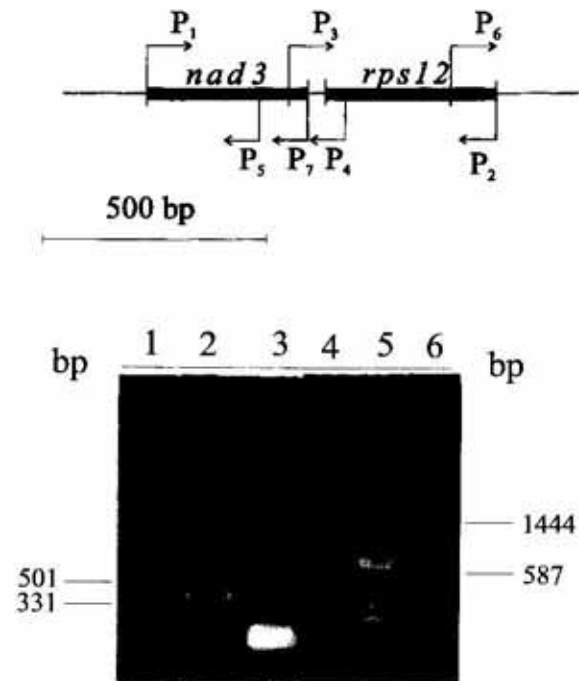
The arrangement and conservation in the nucleotide sequence of the *nad3* and *rps12* genes also suggest their cotranscription. This was confirmed in a few cases, although the amounts of transcripts varied among different plants [3, 9, 11]. In view of some differences in the nucleotide structure of the *nad3* and *rps12* genes [10, 12], we decided to characterize the transcripts of these genes in six-day-old *L. luteus* seedlings. As is shown in Fig. 1,



**Figure 1.** Northern blot analysis of the *nad3*, *rps12* and linked *nad3-rps12* genes from *L. luteus*.

In lane **A** the blot was tested with the linked *nad3-rps12* genes; in lane **B** with the *nad3* gene; in lane **C** with the *rps12* gene.

Northern blot analysis revealed three transcripts of about 1.80 kb and 2.95 kb and 1.4 kb in size for the *nad3* gene, the *rps12* gene and for the whole *nad3-rps12* unit, respectively. These values are very similar to the results obtained by other authors working with seed plants [3]. The results shown in Fig. 1 indicate that lupin *nad3* and *rps12* genes are cotranscribed, because transcripts long enough (2.95, 1.8, 1.4 kb) to include two coding se-



**Figure 2.** RT-PCR analysis of *nad3-rps12* cotranscription.

cDNA amplification products stained by ethidium bromide. Lane 1 is the molecular size marker: pUC19 digested with *MspI* (501/489; 404; 331; 242; 190; 147 bp fragments). Lane 2 contains the positive control of PCR (*nad3* gene amplified with  $P_1$  and  $P_7$  primers). The amplification in lane 3 was obtained with  $P_1$  and  $P_7$  primers. Lane 4 is the positive control of PCR (*nad3-rps12* genes amplified with  $P_1$  and  $P_2$  primers). The product in lane 5 was obtained with  $P_1$  and  $P_2$  primers. Lane 6 contains the molecular size marker: pUC 19 digested with *HaeIII* and *TaqI* (1444; 736; 587; 458; 434; 298; 267/257; 174; 102 bp fragments). In both cases negative control reactions without reverse transcriptase or without RNA were performed. The positions of primers:  $P_3$ ,  $P_4$ ,  $P_5$  and  $P_6$  used for sequencing are indicated above.

quences can be detected among the hybridizing bands. The intensity of the 1.80 kb transcript signal is generally stronger than that for 2.95 kb and 1.4 kb, probably because of the velocity of pre-mRNA processing.

We also analysed the transcripts of *nad3* gene and *nad3-rps12* genes by RT-PCR. To establish cotranscription of two genes, cDNA analysis from the cotranscript of the *nad3-rps12* was performed with two primers: P<sub>2</sub> and P<sub>7</sub>. The P<sub>2</sub> primer corresponds to the end of the *rps12* gene, while P<sub>7</sub> primer to the end of *nad3* gene. The obtained cDNA was amplified using the P<sub>1</sub> and P<sub>2</sub> and also P<sub>1</sub> and P<sub>7</sub> primers. Figure 2 shows the results of this examination. Here again, the obtained values of over 357 bp for the *nad3* gene as well as about 783 bp for the *nad3-rps12* genes strongly suggest the cotranscription of these genes. The identities of RT-PCR products were confirmed by restriction analysis (not shown).

All the presented data confirm for the first time that the nucleotide sequence and the pattern of transcription of linked *nad3* and *rps12* genes are strongly conserved also in the family *Fabaceae*.

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