

Lupin *nad9* and *nad6* genes and their expression: 5' termini of the *nad9* gene transcripts differentiate lupin species

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Abstract

The mitochondrial *nad9* and *nad6* genes were analyzed in four lupin species: *Lupinus luteus*, *Lupinus angustifolius*, *Lupinus albus* and *Lupinus mutabilis*. The nucleotide sequence of these genes confirmed their high conservation, however, higher number of nucleotide substitution was observed in the *L. albus* genes. Southern hybridizations confirmed the presence of single copy number of these genes in *L. luteus*, *L. albus* and *L. angustifolius*. The expression of *nad9* and *nad6* genes was analyzed by Northern in different tissue types of analyzed lupin species. Transcription analyses of the two *nad* genes displayed single predominant mRNA species of about 0.6 kb in *L. luteus* and *L. angustifolius*. The *L. albus* transcripts were larger in size. The *nad9* and *nad6* transcripts were modified by RNA editing at 8 and 11 positions, in *L. luteus* and *L. angustifolius*, respectively. The gene order, *rps3-rpl16-nad9*, found in *Arabidopsis thaliana* is also conserved in *L. luteus* and *L. angustifolius* mitochondria. *L. luteus* and *L. angustifolius* showed some variability in the sequence of the *nad9* promoter region. The last feature along with the differences observed in *nad9* mRNA 5' termini of two lupins differentiate *L. luteus* and *L. angustifolius* species.

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1. Introduction

Mitochondria contain several large multisubunit enzymatic complexes that are composed of proteins encoded in the mitochondrial and nuclear genomes. Higher plant NADH dehydrogenase (NADH: ubiquinone oxidoreductase; complex I) of the inner mitochondrial membrane

consists of approximately 32 subunits (Herz et al., 1994). Among them, only nine subunits are encoded in the mitochondrial DNA (mt DNA).

In higher plants, mitochondrial genes of complex I subunits are scattered throughout mtDNA. The complete sequence analysis of *Arabidopsis thaliana*, as well as *Beta vulgaris* and *Oryza sativa* mtDNAs (Unselde et al., 1997; Kubo et al., 2000; Notsu et al., 2002) revealed that the location of the genes for subunits of complex I between these three genomes was different. This implied that *nad* genes would be involved in different transcription/cotranscription units. Most of the *nad* genes are involved in cotranscription with other genes (Giegé et al., 2000; Haouazine et al., 1993; Itchoda et al., 2002). In addition, the fact that mitochondrial genomes show high complexity resulting from the varying number of recombinationally active repeats would also result in flexible locations of mtDNA segments containing *nad* genes.

Most of the plant mitochondrial genes identified so far are uninterrupted reading frames (ORFs), however, some

Abbreviations: A, adenosine; aa, amino acid(s); bp, base pair(s); C, cytidine; cDNA, DNA complementary to RNA; D, darkness (in photoperiod); d, deoxyribo; DNase, deoxyribonuclease; dNTP, deoxyribonucleoside triphosphate; G, guanosine; kb, kilobase(s) or 1000 bp; L, light (in photoperiod); mRNA, messenger RNA; mt, mitochondrial; *nad*, mitochondrial genes for subunits of NADH dehydrogenase; NADH, reduced form of the nicotinamide-adenine dinucleotide; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; R, purine; RNase, ribonuclease; *rpl16*, gene for ribosomal protein L16; *rps3*, gene for ribosomal protein S3; RT-PCR, reverse transcription-PCR; SDS, sodium dodecyl sulfate; ss, single strand(ed); SSC, 0.15 M NaCl/0.015 M Na₃ citrate (pH 7.6); T, thymidine; TIS, transcription initiation site(s); u, unit(s); U, uridine.

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nad genes are split by introns. Their exons are sometimes distributed over a few distant and independently transcribed genomic locations. To join the different mRNA molecules for ORFs, *trans*-splicing events are required. Nevertheless, among *nad* genes, one can distinguish some intronless ORFs, such as *nad9* and *nad6*.

Furthermore, for the maturation of the *nad* transcripts, the editing of RNA is also required (Maier et al., 1996). Editing has been observed in all major groups of land plants, but not in algae (Hiesel et al., 1994). RNA editing in plant mitochondria modifies the plant mitochondrial primary coding information context with numerous C–U transitions and some rare reverse events. The abundance of editing sites in plant mitochondrial transcripts appears to be one of the greatest, although there is no clear influence of these events on the steady-state transcript level. The ORFs for all subunits of the NADH: ubiquinone oxidoreductase have been shown to belong to gene groups that are edited at higher frequencies than other gene groups. Similar frequency distributions have been observed in some plant species, but not in others (Giegé and Brennicke, 1999).

In higher plants, transcription of mitochondrial genes starts frequently at multiple sites from promoter regions that are probably recognized by phage-type mitochondrial RNA polymerases. The alignment of sequences surrounding transcription initiation sites led to the identification, both in monocots and dicots, the conserved motifs having a core element 5' CRTA 3'. In the mitochondrial promoters of *nad* genes, different motifs as well as a promoter without any motifs were identified.

Previously, we analyzed the structure and arrangement of the lupin *nad3* gene linked with the *rps12* gene, as well as their expression (Rurek et al., 1998). In the present study, we characterized the *nad9* and *nad6* genes of four lupin species. Although the *nad9* and *nad6* genes were analyzed in seven higher plant species (GenBank data), the data concerning transcripts are rather limited. To gain more information about the *nad9* and *nad6* genes belonging to one family, some lupin species as representatives of the *Fabaceae* were taken for analysis. To our knowledge, such studies had not been performed before. Lupins are among the most important grain legumes cultivated worldwide for their protein-rich seeds and the oldest genome in evolutionary terms. However, as a genus *Lupinus*, it needs additional molecular characterization in order to reconstruct the evolutionary history of some distinct groups (Käss and Wink, 1997).

We now provide new comparative information about *nad* genes and the new insight into their expression.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *Lupinus luteus* (cv. Radames) and *Lupinus angustifolius* (cv. Mirela) were provided by the Plant Breeding

Station at Wiatrowo (Poland). Seeds of *Lupinus albus* (cv. Wat) were provided by the Plant Breeding Station at Przebędowo (Poland). Seeds of *Lupinus mutabilis* 'Sweet' (mutant line KW) were obtained from E. Sawicka-Sienkiewicz (Wrocław, Poland) and calli of *L. mutabilis* from J. Rybczyński (Warsaw). *L. albus* suspension culture was a gift of P. Wojtaszek (Poznań).

Prior to imbibition, seeds were surface sterilized in 0.7% (w/v) calcium hypochlorite for 15 min and then grown in darkness at 23 °C for 6 days for the extraction of mtDNA or RNA, but 3 days for extraction of total DNA. For isolation of mtRNA from green hypocotyls, lupin was grown in darkness for 3 days at 25 °C and was then transferred to light (45 µmol/m²/s, photoperiod: 12L/12D) for 3 days.

2.2. Isolation of mitochondria

Mitochondria were isolated from a 6-day old etiolated lupin hypocotyls and roots. After differential centrifugation, mitochondria were further purified through 0.6–1.8 M discontinuous sucrose gradients (Karpińska and Augustyniak, 1989). Mitochondria used for isolation of RNA were not treated with DNase I.

2.3. Nucleic acids extraction

Total DNA from etiolated lupin seedlings was prepared either by "FloraClean" (Bio 101) or "Nucleon" (Amersham-Pharmacia) plant DNA isolation kits. MtDNA and mtRNA were prepared, as described previously (Rurek et al., 1998).

Total RNA from lupin calli and cell suspension cultures was prepared following a standard protocol (Chomczyński and Sacchi, 1987).

2.4. DNA and RNA gel blot analyses

MtDNA (3 µg) were digested with *EcoRI*, *HindIII* or *BamHI* restriction enzymes, fractionated on a 0.8% agarose gel and Southern blotted onto a Hybond-N membrane (Amersham-Pharmacia). Hybridizations were carried out at 42 °C for 18 h in a mixture of 50% formamide, 10× Denhardt solution, 5×SSC, 0.1% SDS, 1 mM EDTA, pH 8.0 and 200 µg/ml salmon sperm DNA. The membrane was washed once in 2×SSC, 0.1% SDS at room temperature for 5 min, three times in the same solution at room temperature for 20 min, then once in 0.1×SSC, 0.1% SSC at 42 °C for 1 h, and finally, in 2×SSC, 0.1% SDS at room temperature for 10 min.

Northern blot analysis was carried out, as described previously (Rurek et al., 1998), using either 13.5 µg of total mtRNA or 50 µg of total cellular RNA.

The probes were labelled using the "Multiprime DNA Labelling System" (Amersham-Pharmacia) according to the manufacturer's instructions. The probes were generated

by PCR (all the primers used in experiments are listed in Table 1).

2.5. PCR amplification and cDNA synthesis

The amplification of gene specific fragments and short 5' flanking sequences containing promoters was carried out in a mixture containing in 50 µl the following components: 20 ng of mitochondrial or 50 ng of total DNA, 10 mM Tris–HCl, pH 8.8, 50 mM KCl, 0.1% (v/v) Triton X-100, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.45 µM of each primer and 1 u of DyNAzyme II DNA polymerase (Polygen, Poland). The following primer pairs were used for the amplification of *nad9* and *nad6* genes: ND9-1for/ND9-2rev, ND9-4for/ND9-5rev and ND6-1for/ND6-2rev, respectively. Cycle times were as follows: denaturation for 7 min at 97 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C, and the last elongation step of 10 min at 72 °C.

The primers for amplification of *rps3* and *rpl16* genes were as follows: RPS3for/RPS3rev and RPL16for/RPL16rev. These primers were designated on *Arabidopsis* mtDNA sequence (Accession number: NC 00 1284, nucleotide positions: 26160–26996 and 25153–25567). The annealing temperature in this amplification was lowered to 53 °C. In the case of amplification of *nad9* 5' flanking regions with the primers PROM-NAD9for/ND9-2rev and PROM-NAD9for/PROM-PE1rev, the PCR thermal profile was changed as follows: the time of denaturation and annealing was shortened to 45 s, the annealing temperature was lowered to 51 °C, and the reaction was conducted in the presence of 1 mM spermidin (Sigma).

Long-range PCR amplification of the 4 kb region covering *rpl16* and *nad9* genes was performed with primers

RPL16for/ND9-2rev. The assay mixture contained in 50 µl: 100 ng of mitochondrial or 800 ng of total cellular DNA, 50 mM Tris–HCl, pH 9.0, 15 mM (NH₄)₂SO₄, 0.1% (v/v) Triton X-100, 2 mM MgCl₂, 0.4 mM of each dNTP, 0.8 µM of each primer and 2.5 u of DyNAzyme EXT DNA polymerase (Polygen, Poland). Cycle times were as follows: denaturation for 3 min at 94 °C, followed by 11 cycles of 10 s at 92 °C, 30 s at 56 °C, and 7 min at 68 °C. One minute and 10 s were added to elongation steps every 5th cycle in the next 20 cycles. In the last cycle the elongation stage lasted 15 min.

First-strand cDNA synthesis was performed on 3.5–5 µg total mtRNA from each of three lupin species as previously described (Rurek et al., 1998). The resulting cDNAs were amplified by PCR with primers ND9-1for/ND9-2rev for *nad9* and ND6-1for/ND6-2rev for *nad6*, respectively. In addition, a reaction without reverse transcriptase was included as a control for each cDNA first-strand synthesis. The PCR control gave no amplified fragments indicating that mtRNA preparations were free of the contaminating mtDNA.

PCR and RT-PCR products were analyzed on 0.8–1.2% agarose gels. The respective fragments were purified from of gel with the Qiaex II Gel Extraction Kit (Qiagen).

2.6. Cloning and sequence analysis

Gel-purified products were cloned in pGEM-T Easy Vector System II (Promega), according to the manufacturer's instructions. Transformation of *E. coli* TG1 competent cells and screening of recombinant colonies were carried out following standard protocols (Sambrook et al., 1989). Cloned DNA and cDNA fragments were sequenced on both strands by the dideoxy chain termination method with an automated DNA sequencer (ABIPrism, Perkin-Elmer Applied Biosystems). DNA sequencing data were analyzed with BLASTN and CLUSTAL algorithms (Altschul et al., 1997). The primers used for sequencing analyses (denoted by an asterisk* in Table 1) were designed on pUC and M13 vector systems as well as based on the *nad9* and *nad6* sequences of wheat and *B. vulgaris*, respectively. The primers used for amplification and sequencing of 5' flanking regions of the genes were designed on the proper sequences of *A. thaliana* and *B. vulgaris*.

2.7. Restriction analysis

The *rpl16-nad9* intergenic region of *L. luteus* and *L. angustifolius* was digested with the following restriction enzymes: *Hind*III, *Rsa*I and *Dde*I. The restriction patterns were analyzed on agarose and acrylamide gels.

2.8. Primer extension analysis

Primer extension analysis was carried out using 7 µg of total mtRNA and 5' end-labelled primers (specific activity:

Table 1
Oligonucleotides used for amplification, cDNA synthesis, sequencing and primer extension

Primer	Sequence (5'–3')
ND9-1for*	ATTGGAAGAGAAGAAGCGGAACT
ND9-2rev*	AGCATTTCCTATTGATTGTGCC
ND9-4for	ATGGATAACCAATCCATTTTCCAATA
ND9-5rev	TTATCCGTCGCTACGCTGTCCCAAG
ND9-6for*	ACTGACTACCCATTTCCATTGT
ND9-7rev*	TTATGGACAACCTCAAATCTTC
PROM-NAD9for*	ATGAAGATAAAGTGTGGAAAGATCT
PROM-PE1rev*	ACCCATTTCTTGGGTAAAGTCTCCC
PROM-PE2rev	TCTTTCCATTTTGTTTACCCATTTTC
ND6-1for*	ATGATACTTTCTGTTTTGTCTGAGCCC
ND6-2rev*	TTAGTAGATCGTGAGTGGGTCAGTCG
PE1-NAD6rev	GTGTCGCGAAAGACTGGGATGG
PE2-NAD6rev	AGCGGAAAATCGAGACCTAACA
pUC/M13for*	CGCCAGGGTTTTCCAGTCACGAC
pUC/M13rev*	CAGGAAACAGCTATGAC
RPS3for	TTCGGCTTTCGTCTCGGTAGG
RPS3rev	CGAGATGGTGGTTTTAGTTGT
RPL16for	GTTACGGAATCTCAGGTGTCA
RPL16rev	AACTCACACCATCCATTTCAA

An asterisk (*) indicates primers used for sequencing analyses.

3×10^6 cpm/pmol) for each experiment. Labelling of primers at their 5' ends was performed with T4 polynucleotide kinase (Promega) using the buffer and protocol supplied by the manufacturer. The extension reaction was carried out using 100 units of M-MLV reverse transcriptase (Promega) for 30 min at 37 °C. Sequences of primers used for extension analysis (PROM-PE1rev, PROM-PE2rev, PE1-NAD6rev and PE2-NAD6rev) are listed in Table 1.

3. Results

3.1. Characterization of the lupin genomic and cDNA *nad9* and *nad6* sequences

In order to investigate the copy number of the *nad9* gene in lupins, a PCR amplified *L. luteus nad9* gene obtained with ND9-1for/ND9-2rev primers (Table 1) was used as a probe to hybridize a Southern blot of *Hind*III, *Eco*RI and *Bam*HI restricted *L. luteus* and *L. angustifolius* mtDNAs. The probe hybridized to only a single band indicating its specificity to the unique genomic sequence context in both investigated lupins (data not shown). The same experiment was performed for determination of the *nad6* copy gene.

L. luteus probe amplified with ND6-1for/ND6-2rev primers (Table 1) was used to hybridize a Southern blot of *L. luteus*, *L. angustifolius* and *L. albus* mtDNAs restricted with the same enzymes as indicated for the *nad9* gene. This analysis also confirmed that *nad6* gene was present in one copy in three analyzed lupins (data not shown).

The mitochondrial or total cellular DNAs of four lupin species were used for the amplification of the *nad9* and *nad6* genes. Three independent PCR amplifications, cloning and sequence analyses were conducted in the each case.

Sequence analysis revealed an open reading frame (ORF) of 573 bp for the *nad9* gene and an ORF of 618 bp as the *nad6* gene, respectively. The nucleotide sequence of the *nad9* gene is deposited in the GenBank databases: *L. luteus*—Accession number: AF279444), *L. angustifolius*—AF279445, *L. albus*—AF279444 and *L. mutabilis*—AF279443. The *nad9* genes of *L. luteus* and *L. angustifolius* were identical. The *nad6* gene sequence determined in *L. luteus*—Accession number: AF279439, *L. angustifolius*—AF279440, *L. albus*—AY134476 and *L. mutabilis*—AF279441 species revealed that the sequences in *L. luteus* and *L. mutabilis* were identical. In both sequenced genes, no introns were detected.

The comparison of the *nad9* and *nad6* sequences in four lupin species also revealed, that among *nad9* genes, there were only one or two nucleotide substitutions, while among the *nad6* genes—the 3 up to 6 substitutions were noticed. It is interesting to note that the 356-bp long 5' flanking regions of the *L. luteus* and *L. angustifolius nad6* genes were identical, although the 321-bp long 5' flanking region of the *nad9* gene of *L. luteus* contained a 6-nt insertion and two

substitutions in comparison with the 209-bp long sequence of *L. angustifolius*.

To determine the extent of RNA editing in the *nad9* and *nad6* transcripts, cDNAs covering the coding regions were obtained by RT-PCR using primers: ND9-2rev/ND9-1for and ND6-2rev/ND6-1for, respectively (Table 1). The sequence of the four independent *nad9* cDNA clones for each lupin revealed that editing occurred at 8 and 7 sites for *L. luteus* and *L. angustifolius*, while *nad6* cDNAs in *L. luteus* showed 11 sites (*nad9* cDNAs accession numbers: AF279447, AF279449, *nad6* cDNAs accession number: AF279442). Differences in the editing pattern between analyzed lupin species of *nad9* and *nad6* transcripts were not significant (Fig. 1) and showed conservation in C to U conversion type. All those alterations were not silent and occupied the first or second codon positions. In the case of

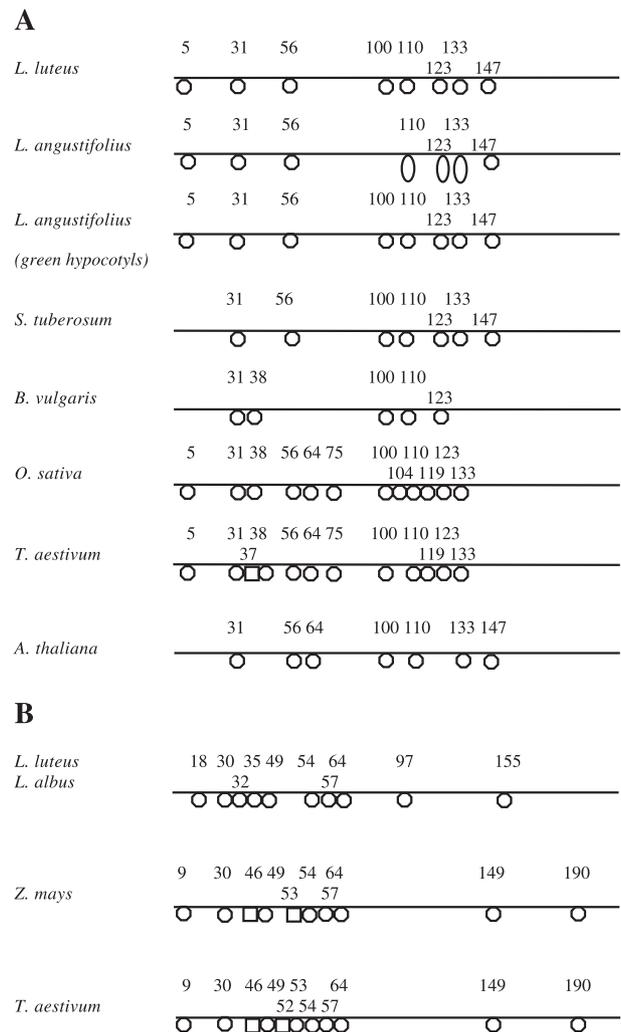


Fig. 1. Alignment of the *nad9* (A) and *nad6* (B) transcript editing patterns of the analyzed lupin species and other plants. Alterations that change a codon to a non-synonymous form are represented by a circle. Silent changes are indicated by a square. Three partially edited codons of *L. angustifolius nad9* mRNAs isolated from etiolated hypocotyls are denoted by ellipsoids.

codon no. 30 in the *nad6* transcript, the editing altered both the first and the second positions. In the case of etiolated hypocotyls of *L. angustifolius*, contrary to green material, partial editing of codon nos. 110, 123 and 133 was observed (Fig. 1). On the whole, the NAD9 and NAD6 lupin subunits of lupin complex I were altered by editing in their overall amino-acid composition by 4.2% and 4.8%, respectively.

3.2. The organization of the lupin mitochondrial *nad9* gene

It has been reported that the close spatial arrangement of the *nad9* gene of *A. thaliana* with ribosomal protein genes led to their cotranscription (Giegé et al., 2000). To study the organization of the *nad9* gene in two analyzed lupin species, restriction digestion of long-range PCR products as well as the Southern hybridization with the *rps3*, *rpl16* gene-specific probes were carried out. The relative intensity of hybridization signals suggested the presence of a single copy for both genes in the mitochondrial genomes of the two lupins. Those analyses also confirmed that the gene order was identical to that known from *A. thaliana*: the *rps3* gene was mapped on the 4.5 and 2 kb *HindIII*, 11.2 kb *EcoRI* and 6.3 kb *BamHI* mtDNA fragments, and the gene *rpl16* on the 2 kb *HindIII*, 2.4 kb *EcoRI* and 2.3 kb *BamHI* restriction fragments (data not shown).

We also used the long-range PCR amplification of a 4-kb mtDNA fragment of *L. luteus* and *L. angustifolius*, respectively, which covers *rpl16* and *nad9* gene sequences with the idea to map the intergenic region (Fig. 2A). When the 4 kb mtDNA fragment was digested with *HindIII*, *RsaI* and *DdeI*, no differences in the location of restriction sites were observed for the analyzed enzymes between the two lupin species (Fig. 2B).

Taken together, all these analyses suggested that *rps3* and *rpl16* genes were located upstream of the *nad9* gene, both in *L. luteus* and in *L. angustifolius*.

3.3. Detection of the *nad9* and *nad6* transcripts in lupin mitochondria

In order to examine the possibility of cotranscription of the *nad9* gene with *rpl16* and *rps3* genes in two analyzed lupin species, Northern hybridization was performed using the mtRNA extracted from hypocotyls and roots and the *rps3*, *rpl16* and *nad9* gene specific probes. When the *rps3* probe was used for hybridization, two transcripts of about 3.5 and 2.2 kb in size were detected (Fig. 3A). One may calculate that because the sum of length of the second *rps3* exon and *rpl16* gene is approximately 2.5 kb, it was possible that 2.2 kb transcript might be the cotranscript of *rps3* exon

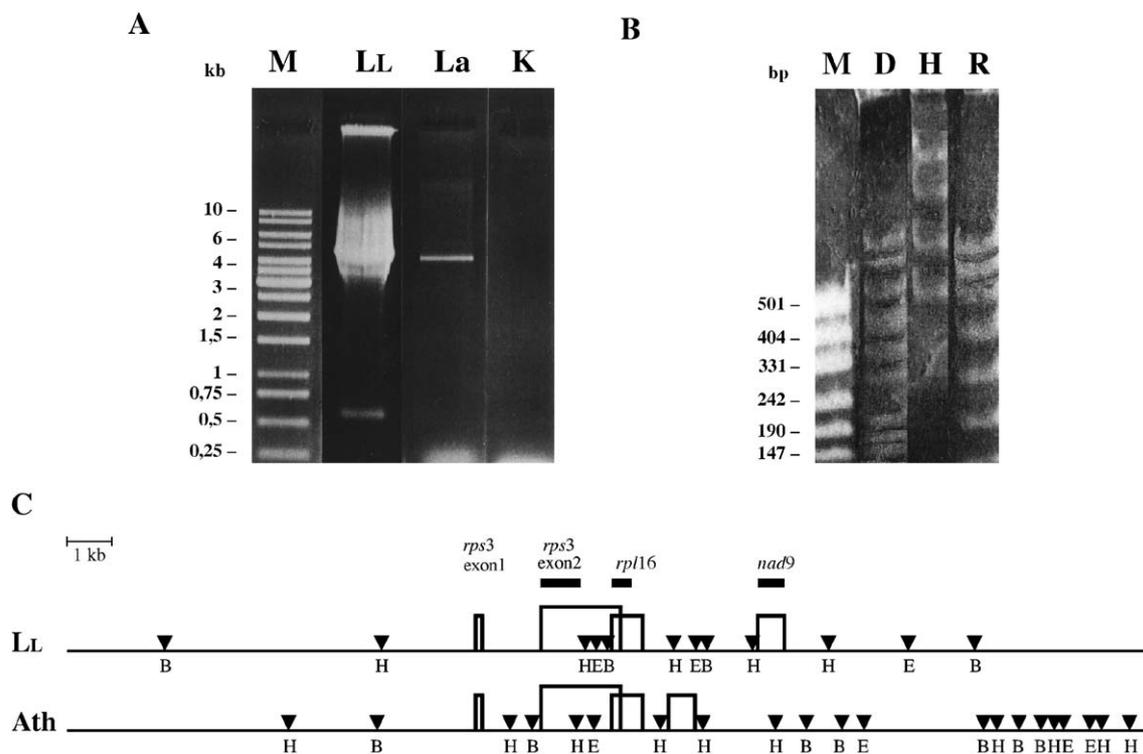


Fig. 2. (A) Long-range amplification of a 4-kb fragment of *L. luteus* (LL) and *L. angustifolius* (La) mtDNAs. Lane K indicates PCR negative control (without nucleic acids). The marker (M), indicated on the left, is a 1-kb DNA Ladder from Fermentas. Products of the amplification were subjected to electrophoresis on a 0.8% agarose gel. (B) Restriction analysis of a *L. luteus* 4 kb mtDNA fragment conducted with *DdeI* (D), *HindIII* (H) and *RsaI* (R). The marker (M) was indicated on the left. (C) A physical map of the mtDNA region including *rps3*, *rpl16* and *nad9* genes in *Arabidopsis* (Ath) and lupin (LL). Restriction sites are indicated for *HindIII* (H), *EcoRI* (E) and *BamHI* (B). The PCR-generated specific probes for these genes are shown by lines below the physical map of the lupin sequence.

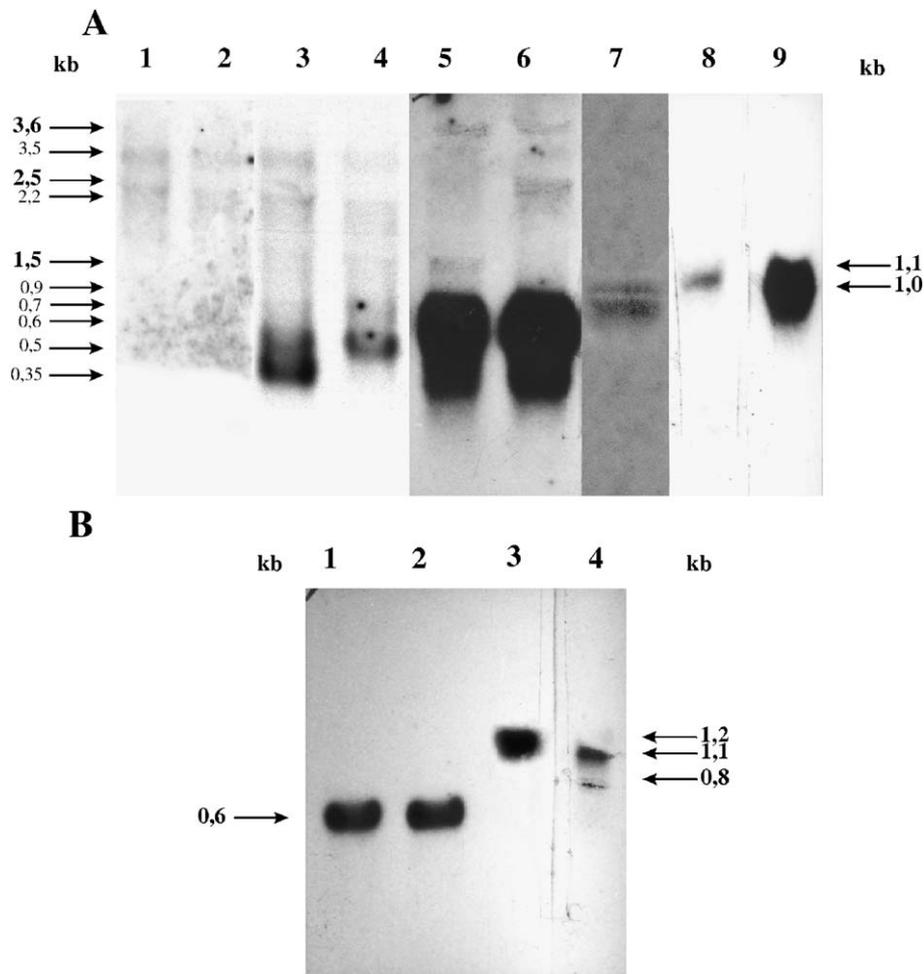


Fig. 3. (A) Northern blot hybridization analysis of the lupin *rps3* (lanes 1 and 2), *rpl16* (lanes 3 and 4) and *nad9* (lanes 5–9) gene transcripts. Lanes: 1, 3 and 5—*L. luteus* hypocotyls and roots mtRNA; 2, 4, 6—*L. angustifolius* hypocotyls and roots mtRNA; 7—*L. albus* hypocotyls and roots mtRNA; 8—*L. mutabilis* calli cellular RNA; 9—*L. albus* suspension culture cellular RNA. (B) Northern blot hybridization analysis of the lupin *nad6* gene transcripts. Lanes: 1—*L. luteus* hypocotyls and roots mtRNA; 2—*L. angustifolius* hypocotyls and roots mtRNA; 3—*L. albus* hypocotyls and roots mtRNA; 4—*L. albus* suspension culture cellular RNA.

and *rpl16* gene. In the case of 3.5 kb transcript, since the sum of length of the *rps3* exons together with intron and *rpl16* gene is approximately 3.3 kb, it was also possible that these transcripts represented cotranscript of two ribosomal protein genes. The pattern of transcripts of the *rpl16* gene probe was similar to that of *rps3* probe; however, apart from the transcripts of 3.5 and 2.2 kb, the small transcript of 0.35 kb was detected in *L. luteus* and 0.5 kb transcript in *L. angustifolius*. Based on these results, it could be concluded that the cotranscripts underwent processing events. When the *nad9* probe hybridized to mtRNA, a single major transcript of about 0.6 kb was detected in *L. luteus* and *L. angustifolius*. Additional minor transcripts of different abundance were also detected in both lupin species. It is interesting to note that in mtRNA of *L. albus* hypocotyls and roots, instead of one main transcript as previously, two transcripts of about 0.9 and 0.7 kb were observed. To compare the transcription pattern in other than hypocotyls and roots types of cells, the total cellular RNAs isolated

from *L. mutabilis* calli and *L. albus* suspension culture were hybridized with a PCR generated probe specific to the *nad9* gene (Fig. 3A). The only single abundant transcripts of about 1 kb were detected in these two species with a relative high accumulation of the *nad9* transcripts in the suspension culture of *L. albus*.

To examine the *nad6* gene expression, Northern blots of mtRNA isolated from *L. luteus*, *L. angustifolius* and *L. albus* hypocotyls and roots as well as Northern blots of total cellular RNA isolated from the suspension culture cells were hybridized with a *nad6*-specific probe. The *nad6* gene is transcribed at a high level, showing a single abundant transcript of about 0.6 kb in *L. luteus* and *L. angustifolius*. In the case of *L. albus* mtRNA isolated from hypocotyls and roots one larger-in size-transcripts of 1.2 kb was detected; however, the most prevalent transcripts detected in the suspension culture were about 0.8 and 1.1 kb (Fig. 3B).

Although the expression of the *nad9* and *nad6* genes was analyzed in different tissues, all results have confirmed the

presence of one main transcript long enough to cover the entire *nad9* and *nad6* coding regions. The differences in the size of transcripts may indicate that different velocity of the processing of primary transcripts occurs in various tissues.

3.4. Determination of the 5' termini of the *nad9* and *nad6* transcripts

The primer extension analysis was performed with two lupin species: *L. luteus* and *L. angustifolius*. The mapping of the 5' termini of the *nad9* transcripts was conducted with two primers: PROM-PE1rev and PROM-PE2rev (Table 1) anchored at 56 and 72 nucleotides downstream of the start codon. In the case of the *nad6* mRNAs, the same analysis was carried out with primers: PE1-NAD6rev and PE2-NAD6rev (Table 1) anchored at 110 and 163 nucleotides downstream of the 5' terminus of the gene. One of the two

oligonucleotides used served as a control of the extension reactions. No differences in the positions of 5' termini for these two primers were observed.

The results of primer extension analyses are shown in Fig. 4A. Two 5' termini of the *nad9* mRNAs were identified both in *L. luteus* and in *L. angustifolius* mitochondria. They were mapped onto residues A at a distance of 116 and 129 bp from the start codon for the *nad9* gene of *L. angustifolius* and residues A and C at a distance of 122 and 273 bp for the *nad9* gene of *L. luteus*, respectively.

The comparison of the relative intensity of *nad9* primer extension products suggested that each of the two extension products in the particular analysis was almost equal in this respect for each lupin.

The two 5' termini of the *nad6* mRNAs were also found in *L. luteus* and *L. angustifolius*. 5' termini of the *nad6* gene mRNAs in these two lupins were mapped about 120 and 40

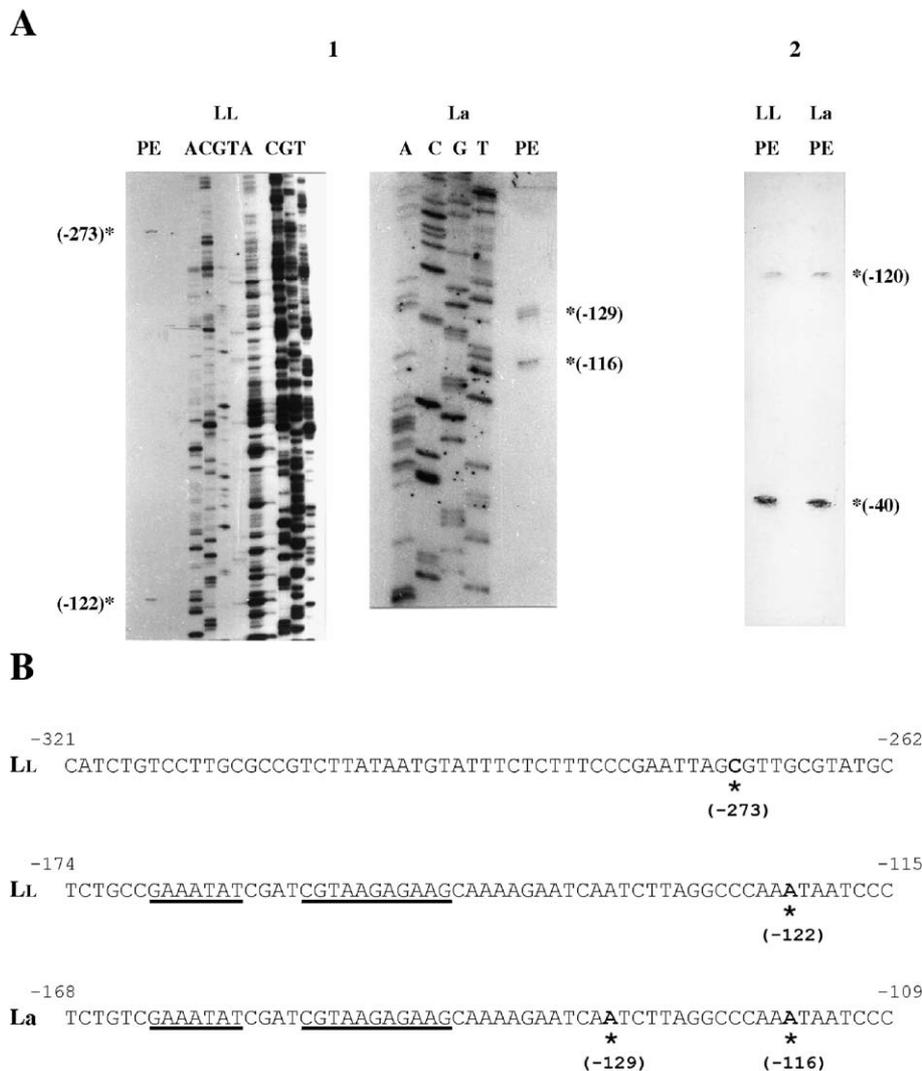


Fig. 4. (A) Detection of the 5' termini of the *nad9* (1) and *nad6* (2) gene transcripts in *L. luteus* (LL) and *L. angustifolius* (La). The extension products are indicated by asterisks. (B) Alignment of the promoter region active in the transcription of the *nad9* gene of two analyzed lupins. The mapped 5' termini are indicated by asterisks. Motifs: CRTA, purine and AT-rich stretches are underlined.

bp from the start codon. This showed that in the case of the *nad6* transcripts, unlike the *nad9* ones, differences in the 5' termini between lupins were not evident.

4. Discussion

The primary goal of this study was to evaluate whether the sequences of some *nad* genes along with promoter regions, or the expression of the *nad* genes, could be useful in distinguishing some lupin species. Although there is a vast body of data concerning *nad* genes from a large number of plant mitochondrial genes that have been characterized up to date, they are poorly understood in some species. In this paper, we present the comparison of the two *nad* genes: *nad9* and *nad6* in some lupin species, including sequence, the transcription pattern, editing, as well as estimation of the positions of the mRNAs 5' termini.

The single content of these genes detected in the analyzed lupins confirmed the data reported in other dicots (Kubo et al., 1993; Grohmann et al., 1994), as well as in monocots (Lamattina et al., 1993; Haouazine et al., 1992; Nishiwaki et al., 1995; Nakazono et al., 1996a,b; Haouazine-Takvorian et al., 1997). In the case of only *nad6* gene, Nugent and Palmer (1993) observations suggested the presence of repeated or disrupted by introns, *nad6* ORF in *Oenothera*, *Pisum* and *Crambe*.

The *nad9* gene sequences in the four lupin species appeared to be highly homologous with known *nad9* of 5 dicots (96–98% identity), such as *Solanum tuberosum*, three species of beet (*B. vulgaris*, *Beta trigyna*, *Beta webbiana*) and *A. thaliana*, and slightly less (95–96%) with monocots, like *Triticum aestivum* and *O. sativa*. The lupin *nad6* gene also shared high identity (93–95%) to the ORFs of other dicots, such as *Helianthus annuus*, *B. vulgaris*, *A. thaliana*, *Brassica campestris* and *Daucus carota*, and monocots of *O. sativa*, *T. aestivum* and *Zea mays*. The similarity of the *nad9* gene with the ORF of *Marchantia polymorpha* was only 78%. The comparison of the *nad9* sequences of four lupin species revealed maximum two or three nucleotide substitutions present in *L. albus*. The same, higher level of substitutions as compared to other lupin species was observed in the *nad6* gene of *L. albus*. These results agreed well with the existence of postulated evolutionarily lines of *Lupinus*: the line containing *L. angustifolius* and *L. luteus* and another line including *L. albus* (Käss and Wink, 1997).

We also tested whether the editing of the *nad9* and *nad6* transcripts could differentiate the analyzed lupins. Although we analyzed a limited number of cDNA clones, we could observe a considerable accumulation of the editing sites in the lupins *nad9* and *nad6* gene transcripts. The editing of lupin *nad9* transcripts resembled, on the whole, that of *S. tuberosum* (Grohmann et al., 1994). However, the editing of *L. luteus* and *L. angustifolius* codon nos. 5 and 100 is typical of monocots, rather than dicots. These alterations

distinguished lupins from *S. tuberosum* and *B. vulgaris* (Fig. 1).

In the case of *L. luteus nad6* mRNA, codon nos. 46, 57, 149 and 190 turned out to be the only ones preedited in this species. The other five codons of the *nad6* gene transcripts preedited in *Z. mays* were found to be edited in *L. luteus* (Haouazine-Takvorian et al., 1997). The characteristic feature of the lupin *nad6* transcripts is the lack of editing of codon no. 9. To summarize, our results have not allowed us to draw the conclusion about differences in editing pattern of the *nad9* and *nad6* mRNAs of the analyzed lupin species. Nevertheless, we obtained some indications of the presence of partial editing in the *nad9* mRNAs codon nos. 110, 123, 133 of *L. angustifolius*, which may be dependent on the tissue and growth conditions.

The next aim was to ascertain whether the expression of *nad9* and *nad6* genes could differentiate some lupin species. Northern blot hybridization was carried out with RNAs isolated from mitochondria of hypocotyls and roots. Due to the data that expression pattern of the *nad9* and *nad6* genes have very often reflected their cotranscription with other genes (Giegé et al., 2000; Haouazine et al., 1993; Haouazine-Takvorian et al., 1997; Itchoda et al., 2002; Nakazono et al., 1996a,b), we have checked the organization of the lupin *nad9* gene in mitochondria of *L. luteus* and *L. angustifolius*. Hybridizations of Southern blots with gene specific probes confirmed that in these two species, the order of the *rps3*, *rpl16* and *nad9* genes was identical to that of *A. thaliana*. It has to note that in *B. vulgaris*, the above-mentioned gene order is altered (Kubo et al., 2000). These experiments as well as the restriction analysis with three enzymes have not allowed us to discover differences in the organization of *L. luteus* and *L. angustifolius rpl16-nad9* intergenic region. In addition, we also noted that in lupins, *A. thaliana*, *Brassica napus*, *Petunia hybrida*, *O. sativa* and *S. tuberosum*, but unlike in *Oenothera* (Bock et al., 1994), the *rps3* and *rpl16* genes overlapped.

The analysis of the distribution of restricted sites in the fragment of lupin mtDNA containing *rps3*, *rpl16* and *nad9* genes displayed the different localization in comparison with *A. thaliana*. In addition, a new BamHI site, absent in the *Arabidopsis* sequence, was located in the *rps3* gene of lupin (Fig. 2C). This site is also present in the respective ORFs of *B. napus*, *P. hybrida* and *S. tuberosum* (GenBank data).

In order to gain insight into the expression of the *nad9*, *rpl16* and *rps3* genes, Northern blot hybridizations were carried out using ribosomal protein and *nad* gene probes. The obtained results seem to indicate that the cotranscription of the only ribosomal protein genes takes place. Lack of signals corresponding to the *nad9* mature transcript on Northern blot hybridized with ribosomal protein gene probes seems to be conclusive in this matter. Moreover, the presence of nine nucleotide consensus sequence with 5'CRTA3' motive upstream of the *nad9* gene as well as the similar sequence motive upstream of *rps3* exons, seems also to favour the existence of separate transcription of the *nad9*.

These results remain, however be confirmed by other methods. Contrary to the most data indicating that the transcription of the *nad9* gene is complex (Giegé et al., 2000; Nakazono et al., 1996a; Lu and Hanson, 1996), lupin *nad9* gene displayed mainly the one very abundant transcript. We also noted that this transcript was not stable because underwent degradation to different extent during the year in both analyzed lupins. This observation was made on lupins grown in identical conditions and mtRNA isolations performed from hypocotyls and roots (data not shown).

The comparison of the *nad9* transcripts detected in lupins hypocotyls and roots mitochondria revealed the difference in size of transcripts. In *L. albus*, instead of 0.6 kb, two transcripts of about 0.7 and 0.9 kb were detected. It is interesting to note that *nad6* gene transcripts detected in hypocotyls and roots mitochondria of *L. luteus* and *L. angustifolius* were the same in size, 0.6 kb, as the *nad9* transcripts. The *nad6* transcript observed in *L. albus* was also larger in size (1.2 kb).

It is claimed (Haouazine et al., 1993) that the use of mRNAs from calli or tissue culture facilitates the detection of intermediate transcripts. Thus, we analyzed the *nad9* and *nad6* transcripts in calli of *L. mutabilis* and suspension culture of *L. albus*. In both tissues, the larger-in size-transcripts were observed (around 1 kb). On the whole, our Northern analyses are in favour of the existence of the simple pattern of transcription of the *nad9* and *nad6* genes in analyzed lupins.

The limited homology of the known promoter region of the *nad9* and *nad6* genes turned our attention to the characterization of the promoter region of these genes in lupins, since the data concerning the promoter structure indicated species-specificity (Hoffmann et al., 1999). In the *nad9* of *L. luteus* and *L. angustifolius* promoter, the consensus sequence with core elements of 5'CRTA3' was observed upstream of the detected 5' termini of the *nad9* transcripts (“–122”, “–129”, Fig. 4B). No such motifs were noticed, however, in the case of one 5' terminus of *L. luteus* (“–273”), which may suggest that different transcription factors are involved in the initiation of the transcription. Based on the data deposited in the GenBank, in the promoter region of the *nad6* gene typical sequence elements 5' CRTA 3' with purine and A/T-rich boxes could be found. The 5' flanking region sequence analysis of the *nad6* *L. luteus* and *L. angustifolius* revealed the presence of such sequence elements (data not shown). Interestingly, these sequences are identical in *L. luteus* and *L. angustifolius*, however, they are different in six nucleotides from *A. thaliana* corresponding region.

A major finding in this work was the detection of differences in the 5' termini of the *nad9* transcripts in *L. luteus* and *L. angustifolius*. For the purposes of this study, we regarded the primer extension analysis as sufficient to reveal the differences in the 5' termini of mRNAs in the analyzed lupins. Other methods, like in vitro 5' capping/

RNase protection or nuclease S1 treatment, or the circularization with ligase RNA, along with the primer extension would better determine the 5' termini, as well as true TISs. Nevertheless, to simplify our analysis, we paid more attention to carrying out of a precise and reproducible primer extension analysis. The three separate analyses for each of the primers were used in the experiments. Additional oligos were introduced to control the reactions. As a result, two 5' termini were mapped for the *L. luteus* and *L. angustifolius* *nad9* and *nad6* gene transcripts.

The first 5' termini of the *nad9* mRNAs were mapped in close vicinity in *L. luteus* and *L. angustifolius* (“–122” in *L. luteus*, “–116” in *L. angustifolius*), due to a 6 nucleotide insertion in the *L. luteus* sequence. The second 5' termini were located in *L. luteus* more distantly (“–273”), than in *L. angustifolius* (“–129”). The very close 5' termini position of the *L. angustifolius* *nad9* mRNAs suggested that the TIS would rely on the promoter motifs located upstream of these termini. It cannot be excluded that some processing and degradation of transcripts took part and marked out the present locations of these two termini.

The 5' termini of the *nad6* transcripts were mapped at 40 and 120 nt upstream of the start codon. About 50 nucleotide upstream of the 5' termini at “–40” 5'AATA3' motive with purines was detected. A similar motive was located about 30 nucleotides upstream of the “–120” termini. The 5' termini determination of the lupin *nad6* transcripts was, to our knowledge, the first example of that kind of analysis for dicot plants. The only known 5' termini of *nad6* mRNAs mapping was conducted in *T. aestivum* (Haouazine et al., 1992), where the terminus was mapped 375 bp upstream of the first ATG codon. The presence of the two 5' termini of the *nad6* transcripts in both investigated lupins may suggest a quite rapid processing of pre-mRNA, if one considers the results of Northern blot hybridizations. The limited data concerning 5' and 3' termini determinations do not allow making correlations of size transcript with Northern data. It must be stressed that the only comparable data exist for the *nad9* and *nad6* transcripts of *T. aestivum* (Lamattina et al., 1993; Haouazine et al., 1993). In this particular case, the correlation was observed for the 0.9 kb *nad9* transcript and the 1.2 kb *nad6* mRNA. Correlation of size transcripts with Northern analysis is also known for the 0.9 kb *nad9* transcript in *S. tuberosum* (Lu and Hanson, 1996). Since no analysis of 3' termini mapping was performed in lupins, we cannot draw any conclusion about the correlation with Northern hybridization results.

Summing up, the different promoter sequence and the 5' termini of the *nad9* mRNAs were the only feature that differentiated the *L. luteus* and *L. angustifolius* species. To confirm the other results indicating that *L. albus* sequence and size of transcripts distinguish this species from the others, the organization of the promoter region and the 5' termini analysis remains to be determined. Nevertheless, the mitochondrial *nad9* and *nad6* genes and the pattern of their expression may be regarded as valuable data in supporting

the postulated phylogeny of *L. luteus*, *L. angustifolius* and *L. albus*.

References

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Bock, H., Brennicke, A., Schuster, W., 1994. *Rps3* and *rpl16* genes do not overlap in *Oenothera mitochondria*: GTG as a potential translation initiation codon in plant mitochondria? *Plant Mol. Biol.* 24, 811–818.
- Chomczyński, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Giegé, P., Brennicke, A., 1999. RNA editing in *Arabidopsis* mitochondria effects 441 C to U changes in ORFs. *Proc. Natl. Acad. Sci. U. S. A.* 96, 15324–15329.
- Giegé, P., Hoffmann, M., Binder, S., Brennicke, A., 2000. RNA degradation buffers asymmetries of transcription in *Arabidopsis* mitochondria. *EMBO Rep.* 1, 164–170.
- Grohmann, L., Thieck, O., Herz, U., Schröder, W., Brennicke, A., 1994. Translation of *nad9* mRNAs in mitochondria from *Solanum tuberosum* in restricted to completely edited transcripts. *Nucleic Acids Res.* 22, 3304–3311.
- Haouazine, N., de Souza, A.P., Jubier, M.F., Lancelin, D., Delcher, E., Lejeune, B., 1992. The wheat mitochondrial genome contains an ORF showing sequence homology to the gene encoding the subunit 6 of the NADH-ubiquinone oxidoreductase. *Plant Mol. Biol.* 20, 395–404.
- Haouazine, N., Takvorian, A., Jubier, M.F., Michel, F., Lejeune, B., 1993. The *nad6* gene and the exon d of *nad1* are co-transcribed in wheat mitochondria. *Curr. Genet.* 24, 533–538.
- Haouazine-Takvorian, N., Takvorian, A., Jubier, M.F., Lejeune, B., 1997. Genes encoding subunit 6 of NADH dehydrogenase and subunit 6 of ATP synthase are co-transcribed in maize mitochondria. *Curr. Genet.* 31, 63–69.
- Herz, U., Schröder, W., Liddel, A., Leaver, C.J., Brennicke, A., Grohmann, L., 1994. Purification of the NADH; ubiquinone oxidoreductase (complex I) of the respiratory chain from the inner mitochondrial membrane of *Solanum tuberosum*. *J. Biol. Chem.* 269, 2263–2269.
- Hiesel, R., Combettes, B., Brennicke, A., 1994. Evidence of RNA editing in mitochondria of all major groups of land plants except the bryophytes. *Proc. Natl. Acad. Sci. U. S. A.* 91, 629–633.
- Hoffmann, M., Dombrowski, S., Guha, C., Binder, S., 1999. Cotranscription of the *rpl5-rps14-cob* gene cluster in pea mitochondria. *Mol. Gen. Genet.* 261, 537–545.
- Itchoda, N., Kubo, T., Estiati, A., Yamamoto, M.P., Handa, H., Mikami, T., 2002. The *rps4* gene in sugar beet mitochondria: insertion/deletion mutations occur within the gene but not disrupt the reading frame. *J. Plant Physiol.* 159, 211–215.
- Karpińska, B., Augustyniak, H., 1989. Lupin chloroplast and mitochondrial tRNA populations and their genes. *Acta Biochim. Pol.* 36, 245–256.
- Käss, E., Wink, M., 1997. Molecular phylogeny and phylogeography of *Lupinus (Leguminosae)* inferred from nucleotide sequences of the *rbcl* gene and ITS 1+2 regions of rDNA. *Pl. Syst. Evol.* 208, 139–167.
- Kubo, T., Mikami, T., Kinoshita, T., 1993. The sugar beet mitochondrial genome contains an ORF sharing sequence homology with the gene for the 30 kDa subunit of bovine mitochondrial complex I. *Mol. Gen. Genet.* 241, 479–482.
- Kubo, T., Nishizawa, S., Sugawara, A., Itchoda, N., Estiati, N., Mikami, T., 2000. The complete nucleotide sequence of the mitochondrial genome of sugar beet (*Beta vulgaris* L.) reveals a novel gene for tRNA^{Cys} (GCA). *Nucleic Acids Res.* 28, 2571–2576.
- Lamattina, L., Gonzalez, D., Gualberto, J., Grienenberger, J.M., 1993. Higher plant mitochondria encode an homologue of the nuclear-encoded 30-kDa subunit of bovine mitochondrial complex I. *Eur. J. Biochem.* 217, 831–838.
- Lu, B., Hanson, M.R., 1996. Fully edited and partially edited *nad9* transcripts differ in size and both are associated with polysomes in potato mitochondria. *Nucleic Acids Res.* 24, 1369–1374.
- Maier, R.M., Zeltz, P., Kössel, H., Bonnard, G., Gualberto, J.M., Grienenberger, J.M., 1996. RNA editing in plant mitochondria and chloroplasts. *Plant Mol. Biol.* 32, 343–365.
- Nakazono, M., Ito, Y., Tsutsumi, N., Hirai, A., 1996a. The gene for a subunit of an ABC-type heme transporter is transcribed together with the gene for subunit 6 of NADH dehydrogenase in rice mitochondria. *Curr. Genet.* 29, 412–416.
- Nakazono, M., Nishiwaki, S., Tsutsumi, N., Hirai, A., 1996b. A chloroplast-derived sequence is utilized as a source of promoter sequences for the gene for subunit 9 of NADH dehydrogenase (*nad9*) in rice mitochondria. *Mol. Gen. Genet.* 252, 371–378.
- Nishiwaki, S., Nakazono, M., Tsutsumi, N., Hirai, A., 1995. Structure of a gene for subunit 9 of NADH dehydrogenase (*nad9*) in rice mitochondria and RNA editing of its transcript. *Plant Cell Physiol.* 36, 1135–1138.
- Notsu, Y., Masood, S., Nishikawa, T., Kubo, N., Akiduki, G., Nakazono, M., Hirai, A., Kadowaki, K., 2002. The complete sequence of the rice (*Oryza sativa* L.) mitochondrial genome: frequent DNA sequence acquisition and loss during the evolution of flowering plants. *Mol. Genet. Genom.* 268, 434–445.
- Nugent, J.M., Palmer, J.D., 1993. Characterization of the *Brassica campestris* mitochondrial gene for subunit six of NADH dehydrogenase: *nad6* is present in the mitochondrion of a wide range of flowering plants. *Curr. Genet.* 23, 148–153.
- Rurek, M., Oczkowski, M., Augustyniak, H., 1998. Conservation of the structure and organisation of lupin mitochondrial *nad3* and *rps12* genes. *Acta Biochim. Pol.* 45, 695–699.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Unsold, M., Marienfeld, J.R., Brandt, P., Brennicke, A., 1997. The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. *Nat. Genet.* 15, 57–61.