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Characterization of *Lupinus luteus* Chloroplast Gene Coding for Components of a Chloroplastic NADH Dehydrogenase

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Abstract

The plastid genomes of several plants contain *ndh* genes homologues of genes encoding subunits of the mitochondrial complex I. We sequenced the part of lupin *ndh*B, *ndh*D and *ndh*F genes in order to compare the structure of these genes with those of *Nicotiana tabacum, Arabidopsis thaliana, Zea mays* and *Oryza sativa* with the idea to detect the presence of stretches with identical aminoacid composition. We were only able to find one or two stretches of this kind of about 16 aminoacid- long in the analyzed fragments of the *ndh* genes. The total number of such stretches was different in particular gene products: for *ndh*C 1, *ndh*B 9, *ndh*D 3 and *ndh*F 6. We have also examined the transcription pattern of *ndh*C, *ndh*K, and *ndh*J genes during lupin development. We show that the greatest amount of *ndh*C, *ndh*K, and *ndh*J transcripts are observed in 7- to 14- day- old lupin seedlings.

We also studied the level of transcription of those genes in plants growing at low temperature. All the data confirmed that the abundance of transcription of *ndh*C, *ndh*K, and *ndh*J genes increased under chill conditions. It has to be noted that the level of transcription of the *ndh*C gene was higher than the other genes probably due to higher stability of this transcript.

Introduction

The plastid genomes of several plants contain eleven *ndh* genes, homologous in sequence to the genes of subunits of the mitochondrial NADH- ubiquinone oxidoreductase (complex I) (Berger, 1993). The peptides encoded by these genes are thought to assemble a large protein complex (Ndh complex). The function of the Ndh complex in higher plants is not established, although it has been suggested that it serves as a component of cyclic photosynthetic electron transport or part of a putative respiratory chain in chloroplasts. The respiratory activity (chlororespiration) was demonstrated in the unicellular green alga *Chlamydomonas reinhardtii* (Bennoun, 1982) and in chloroplast membranes of tobacco, barley, pea and sunflower (Garab, 1989; Gruszecki, 1994; Cuello, 1995; Sazanov, 1996; Feild, 1998).

In higher plants the sequences of the *ndh* genes are highly conserved. These genes are clustered and organized in four transcriptional units. We have previously demonstrated that the peculiar feature of *Lupinus luteus ndh*C and *ndh*K gene order is that the two sequences do not overlap (Oczkowski, 1997). The same gene order for these genes has been shown for pea (Elortza, 1999). In addition, we presented evidence that *ndh*C, *ndh*K and *ndh*J genes were co-transcribed in the chloroplasts. In order to better understand the structure and function of some ndh genes, we continued their characterization by sequencing and transcription analysis.

In this paper we present the results of partial analysis of *ndh*B, *ndh*D and *ndh*F genes and evidence that the transcription pattern of the *ndh*C, *ndh*K and *ndh*J genes differs during the development of lupin, and that the transcripts of these genes are more abundant when plants are grown at low temperature.

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Materials and Methods

Plant materials

Seeds of *Lupinus luteus* cv. Topaz were obtained from the Plant Breeding Station at Wiatrowo near Poznan (Poland). Seeds were sterilized in 7% calcium hypochloride solution for 7 minuts before sowing. The plants were grown at low light intensity 30 mM $m^{-2}s^{-1}$ in a 12h: 12h light: dark regime.

Nucleic acid preparation

Chloroplasts, chloroplast DNA, RNA and total cellular RNA were prepared as described previously (Oczkowski, 1997) Chloroplast DNA and RNA were obtained by separate extraction from plant material.

Labelling of DNA, hybridization and DNA sequencing

Probes, labelling of DNA, hybridization and DNA sequencing were performed in the manner outlined previously (Oczkowski, 1997). Screening for *ndh* genes was performed with a tobacco heterologous probe as well as with PCR amplification products.

RNA analysis

Conditions for Northern blot analysis were the same as described previously (Oczkowski, 1997), with the exception that the amount of total RNA used for reaction in the case of developmental analysis increased to 60 micrograms. For dot- blot analysis 10 micrograms of total RNA denatured in formamide were spotted on the Hybond N (Amersham) membrane by dot-blotter (Biometra).

PCR amplification

The PCR reaction mixture contained the following components in 20 microlitres: 50 ng of DNA, 0.5 mM of each primer, 200 mM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris/HCl pH 8.8, 50 mM KCl, 0.1 % (v/v) Triton X-100, and 1 unit of Taq DNA polymerase (PrimeZyme). The thermal profile included: denaturation at 98℃ for 5 min., followed by 35 cycles: annealing 55℃ 1 min., elongation 72℃- 1 min., denaturation 95℃ 1 min. The reaction was ended by elongation at 72℃ for 10 min.

Primers used for amplification of the *ndh*B fragment were as follows:
P8: 5' CTT GGT TTC GTC CAG TCA TTA 3'
P9: 5' ATT CCT TTT TAT TTC TCA TCA 3'

Sequence data analysis

DNAsis and BLAST programs were used in nucleotide and protein analysis.

The hydropathic profiles of proteins were calculated by using Kyte- Doolittle values of aminoacid (http:// bioinformatics. weizmann.ac.il/hydroph, Kyte , 1982).

Results

In an attempt to extend our knowledge about the structure and function of the *ndh* genes, we screened the lupin chloroplast clone bank for the presence of *ndh*F, *ndh*D and *ndh*E genes. Colony hybridization was conducted with ^{32}P- labelled tobacco probes. Selected clones, designated 92 containing a 5 kb insert and 83 containing a 3 kb insert, were taken for further analysis. Sequencing analysis confirmed that the insert of clone 92 included whole *ndh*D and *ndh*E genes, but clone 83 only the 5' part of the *ndh*F gene of about 0.9 kb.

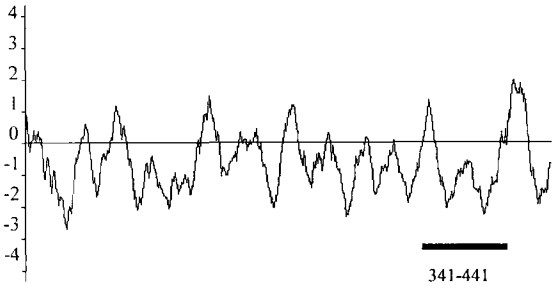
For the purpose of comparison of ndh genes having hydrophobic character, we amplified a 526 bp fragment of the *ndh*B gene including part of exon 2 of this gene.

In view of the fact that the sequences of *ndh* genes are highly conservative we sequenced only parts of these genes to determine the nucleotide sequence identity with *ndh* genes of four other species, as well as to choose fragments possessing the identical aminoacid composition in all these genes.

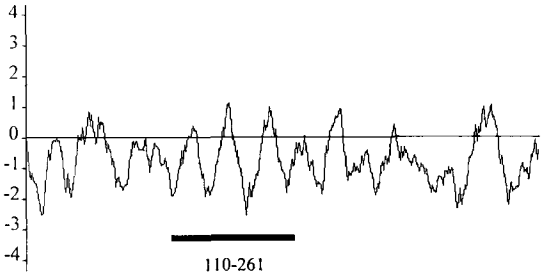
A 259 bp sequence of the lupin *ndh*D gene which corresponds to a tobacco sequence from 755 to 1034 nucleotides showed 85.1 % identity with those of *Nicotiana tabacum, Arabidopsis thaliana, Zea mays* and *Oryza sativa*. A 215 bp sequence of the lupin *ndh*F gene which corresponds to a tobacco sequence from 1161 to 1376 nucleotides showed 67.7 % identity with *Nicotiana tabacum, Arabidopsis thaliana, Zea mays* and *Oryza sativa* genes, and a 299 bp fragment of the lupin *ndh*B gene sequence which corresponds to a tobacco sequence from 199 to 498 nucleotides showed sequence identity with the four above- mentioned sequences of 78.7 %.

All the sequenced fragments of the *ndh* genes were evaluated for stretches with an identical aminoacid composition. We were only able to find one or two long enough stretches of this kind in the analyzed fragments of the *ndh* genes. The comparison of about 16- aminoacid- long stretches coming from each analyzed *ndh* gene is presented in Figure 1.

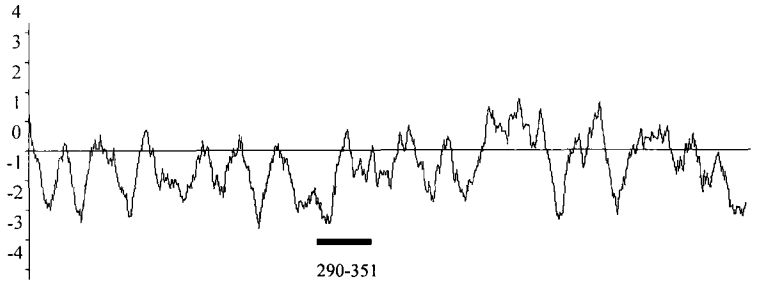
As can be seen, this comparison points to the fact that the products of particular fragments of the *ndh*



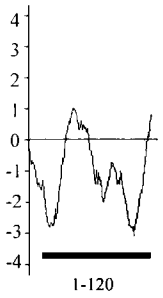
NdhB 510aa(398-411) L A L C L L S L G G L P P L
N.tabacum TTAGCCCTATGCCTCTTATCCCTAGGAGGTCTTCCTCCACTA
L.luteus TTAGCCCTATGTCTCTTATCCCTTGGAGGTCTTCCTCCACTA



NdhD 500aa(149-167) M W G G K K R L Y S A T K F
N.tabacum ATGTGGGGAGGAAAGAAACGTCTGTACTCAGCTACAAAATTT
L.luteus ATGTGGGGGGGGAAGAAACGTTTGTATTCGGCTACAAAGTTT



NdhF 740aa(309-325) A L A Q K D I K R G L A Y S T M S
N.tabacum GCTCTTGCTCAAAAAGACATTAAGAGAGGTTTAGCCTATTCCACAATGTCT
L.luteus GCTCTTGCTCAAAAAGATATTAAGAGAAATTTGGCTTATTCTACAATGTCT



NdhC 120aa(61-79) R Y Y M F A L V F V V F D V E T V F L
N.tabacum CGTTATTATATGTTTGCTCTAGTTTTTGTTGTTTTTGATGTTGAAACGGTTTTTCTT
L.luteus CGTTATTATATGTTTGCTCTAGTTTTTGTTGTTTTTGATGTTGAAACGGTTTTTCTT

Figure 1. Comparison of the sequenced fragments of the lupin *ndh* genes displaying identical amino acid composition with those of *Nicotiana tabacum, Arabidopsis thaliana, Zea mays* and *Oryza sativa*.

genes do not show a predominant amount of one type of aminoacid nor any known aminoacid motifs. Furthermore, we have found no indication that in the whole structure of the four analysed products of the *ndh* genes more than 9 long stretches having 14-19 identical aminoacids exist. The total number of such stretches was different in particular gene products: for *ndh*C1, *ndh*B9, *ndh*D3, and *ndh*F6. This may suggest that only the overall structure of those gene products is important for their function. The function of these gene products known to date is that, they are components of the Ndh complex. If the peptide products of all four analysed *ndh* genes are located in the membrane part of the enzyme as suggested (Berger, 1993), it would be possible for some of them to be exchanged for a similar hydrophobic protein.

We expanded our investigation of the *ndh* genes to their transcription. There is only limited information on the overall transcription pattern of the *ndh* genes during plant development and under the treatment of shock conditions like: light, heat shock, or exposure to low temperature (Du Bell, 1995; Martinez, 1997).

These observations have prompted us to examine the transcription of the *ndh*C, *ndh*K, *ndh*J genes in different stages of lupin development. We studied also the effect of plants growing at low temperature on the transcription of these genes.

The results of transcription analysis in different stages of lupin development are presented in Figure 2.

As shown in Figure 2, dot-blot analysis revealed differences in the level of hybridization with leaf RNA among young and old leaves.

The analysis of the autoradiographs both by visual inspection and scanning densitometry indicated that the generation of a stronger hybridization signal for these *ndh* transcripts is observed in 7- to14- day-old seedlings as compared with 16- to 22- day-old seedlings. The faint signal of *ndh*C gene expression was also observed in 2- day-old seedlings and 4-day-old hypocotyls. The northern analysis performed with these 2-day-old seedlings did not reveal the transcripts, probably because of low stability of the transcript due to a higher velocity of mRNA processing. It has to be noted that 2-day-old lupin seedlings were grown in the dark. The pattern of transcription of the *ndh* genes analysed by northern blot concerns only the *ndh*K and *ndh*C genes. To facilitate a comparison of the transcript size of the *ndh* genes, the same stripped filter was used. This caused the filter used for hybridization with *ndh*J probes to show a very weak signal and therefore is not shown. We also tested the transcription patern in developing

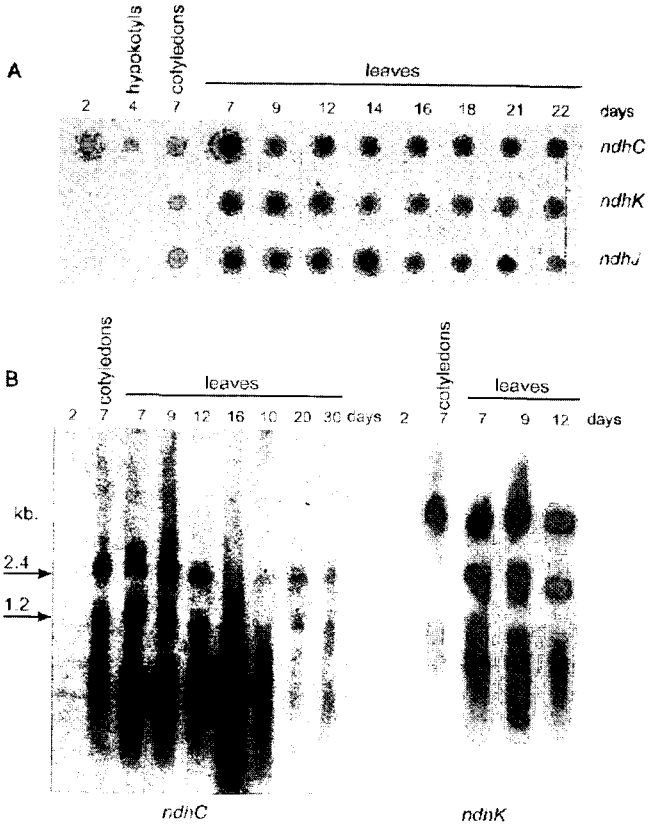


Figure 2. Comparison of the *ndh*C, *ndh*K, *ndh*J gene transcripts in different stages of lupin development by dot-blot (A) and northern analysis (B) Total RNA was isolated from 2-day-old seedlings, 4-day-old hypocotyls, 7-day-old cotyledons, leaves of 7- to 22- day- old seedings and 10-, 20- and 30- day-old pods (time calculated after flowering). The amount of RNA analyzed by dot- blot was evaluated by laser densitometer.

pods. The expression of the *ndh* genes was only detected in 10- day-old pods, which may suggest that the expression of these genes is combined with photosynthesis.

It was possible to see the complex pattern of *ndh* gene transcripts at different stages of lupin development because the gels were overloaded, which caused most of the RNA intermediates to be visible in the process of degradation of full- lenght transcripts.

To confirm that the observed differences were a true reflection of the RNA abundance, we used quantitative RNA dot-blot hybridization as an alternative approach for estimating the relative abundance of the different RNAs. The method is highly reliable in that it is not subject to artifacts resulting from, e.g. variability in the transfer of RNA from gels or effects of RNA abundance on hybridization efficiency.

It has to be noted that the *ndh*C, *ndh*K, *ndh*J tran-

scripts were not detected in roots of lupin plants grown at 25℃. The same was recorded in maize roots (Steinmuller, 1989).

Some information about differences in the relative levels of *ndh* transcripts among different plant organs is also available.When the level of transcription of the *ndh*C, *ndh*K, and *ndh*J genes was analysed in plants grown under chill conditions (4-5 weeks at 4℃), a much stronger hybridization signal was observed than in plants grown at 25℃. The results of those studies are presented in Figure 3.

As is shown, the northern and dot-blot analyses of plants grown at +4℃ display the presence of more abundant transcripts than in control plants. Enhanced levels of the *ndh*C mRNAs are also evident in roots of plants growing for 5 weeks at +4℃. The intensity of *ndh*C transcript signals in roots was

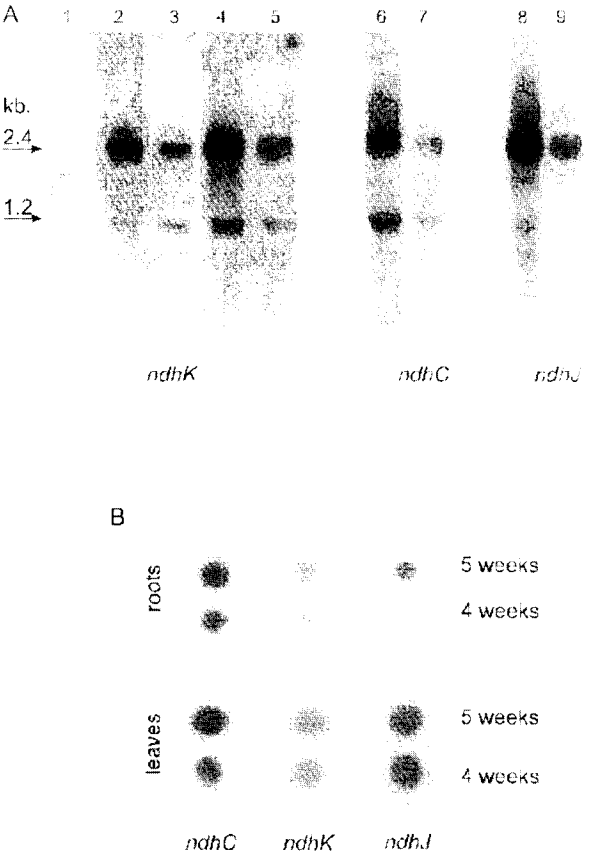


Figure 3. Comparison of the *ndh*C, *ndh*K, and *ndh*J transcript levels in plants grown at 4℃ during 4-5 weeks and plants grown in 25℃. A-northern analysis: 1- total RNA from roots of 5-week-old plants (4℃); 2- total RNA from leaves of 5-week-old plants (4℃); 3- total RNA from plants grown at 25℃; 4, 6, 8- chloroplast RNA isolated from 5-week-old plants (4℃); 5, 7, 9- chloroplast RNA isolated from plants grown at 25℃. B-dot- blot analysis of total RNA from leaves and roots 4-5- week- old plants (4℃) probed with *ndh*C, *ndh*K, and *ndh*J genes.

comparable with the signals in leaves. The more pronounced differences are seen when chloroplast RNA was used for analysis.

It is likely that the transcript levels are governed by a variety of factors, like the regulation at both the transcriptional level and the posttranscriptional level. Greater transcript stability at low temperature as well as promoters responsive to low temperature could also contribute to the observed differences.

The amount of RNA analysed by dot-blot was evaluated by laser- densitometer.

Discussion

Seven plastid *ndh* genes (A, B, C, D, E, F, G) are homologous to hydrophobic subunits of the mitochondrial complex I (Kofer, 1998). According to the model developed for the mitochondrial complex I, the plastid *ndh*A- *ndh*G gene products are located in the membrane- embedded part of the enzyme that is possibly involved in plastoquinone reduction, and the more hydrophilic *ndh*H- *ndh*K gene products form the peripheral arm. Ndh genes are located in a different part of the chloroplast genome: *ndh*C in the large single copy region, *ndh*D and *ndh*F in the small copy region, and *ndh*B in the inverted repeat.

The functional significance of the NDH proteins in higher plants is uncertain. Because of the controversy about the function of the Ndh complex in optimal conditions, it was suggested that the complex would operate probably in stress conditions. It was claimed that in the dark, upon heat stress, the dominant pathway for reduction of the plastoquinone pool was catalyzed by the Ndh complex (Sazanov, 1998). With respect to the potential role of the Ndh complex in photosynthesis, there are some data indicating the participation of this complex in cyclic electron flow around photosystem I in the light and possibly in a chloroplast respiratory chain in the dark (Burrows, 1998; Sazanov, 1998). The action of light was observed for example in pea (Du Bell, 1995), whement, which was comparable w*indh*h the maximum expression of photosynthetic genes. Taken together all the results indicated for dual role of the Ndh complex in the chlororespiratory pathway as well as cyclic photophosphorylation (Sazanov, 1998; Burrows, 1998). The latest evidence points to the role of the Ndh complex in photoprotection (Endo, 1999).

There is no data on how the Ndh complex operates in a chill. Much of the energy leaking in a chill in the light causes the formation of reactive oxygen species, such as superoxide, singlet oxygen, hydrogen peroxide and hydroxyradicals.

We analyzed the effect of a chill on the process of

*ndh*C, *ndh*K, and *ndh*J transcription. The transcript abundance of *ndh*C, *ndh*K, and *ndh*J genes in all tissues examined so far is considered rather low. (Steinmuller, 1989; Matsubayashi, 1987).

In addition, there are some data that northern blot analysis could detect transcripts in the leaves of plants grown in the light and dark. (Matsubayashi, 1987; Steinmuller, 1989) Both our previous and current results on the transcription indicate that for these genes two main transcripts of 2.4 and 1.2 kb are detected also under chill conditions. The transcript of 2.4 kb is long enough to include all three genes.

All the presented data confirm for the first time that the abundance of transcription of *ndh*C, *ndh*K, and *ndh*J genes increase under chill conditions. It will be interesting to examine other related legumes to determine whether a similar pattern of transcription of these *ndh* genes under the same conditions are observed.

In order to clarify the relationship between the transcript accumulation in a chill and the polypeptide accumulation, we are now analysing the pattern of polypeptides of these genes during development.

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