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Immunological and genomical analysis of Trebouxoid phycosymbionts isolated from *Ramalina farinacea* reveals the possible presence of the plastid Ndh complex in lichen algae.

Abstract

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Chloroplasts of most vascular plants contain a NADH dehydrogenase complex (Ndh complex) homologous to the mitochondrial complex I. Eleven *ndh* genes encoding this complex are localized in the chloroplast genome. These genes are not present in the chloroplast genomes of several genera of free-living green algae (*Chlorella, Chlamydomonas*), but they occur in others (*Nephroselmis, Mesostigma, Chaetosphaeridium*). The Ndh complex in vascular plants is involved in the protection against photo-oxidative stress. We have isolated Trebouxoid photobionts from *Ramalina farinacea* L. (Ach.) in order to study the possible presence and function of the Ndh complex in lichenized algae. PCR amplification and sequencing of DNA isolated from purified Trebouxoid photobionts indicates the presence of the *ndh*F gene in these algae. Its expression was confirmed by immunological analysis. The sequence presented a high degree of similarity with the previously described *ndh*D sequence of *Physcomitrella patens* subsp. *patens*, a moss which is used as an alternative model system to investigate the molecular basis in terrestrial plants. The homology was lower when compared with *Anthoceros formosae*, the fern *Huperzia lucidula* or the unicellular green alga *Nephroselmis olivacea*. These results clearly indicate the presence of the *ndh*D gene in Trebouxoid photobionts from *Ramalina farinacea*. They also suggest the possibility of a closer proximity of these Trebouxia plastid genome to the *Bryophytes* and/or the *Pteridophytes* included in the *Lycopodiales* -immediate ancestors of land plants- than to other green algae lineages.

Introduction

Chloroplasts contain a NADH dehydrogenase (Ndh) complex which includes several polypeptides homologous to mitochondrial and eubacterial respiratory complex I subunits. The Ndh complex is composed by at least eleven plastid DNA encoded (*ndh* genes) reading frames (*ndh*A-K) (Sugiura 1992). The function of the Ndh complex in plastids of higher plants is related to protection against (photo)oxidative stress conditions (Martín & al. 1997; Catalá & al. 1997; Casano & al. 1999; Guéra & al. in press). Recent studies have

shown that *ndh* genes are present in most plastid genomes of vascular plants (Hiratsuka & al. 1989; Freyer & al. 1995; Maier & al. 1995). However, they have not been found in the chloroplast genomes of some Gymnosperms (Wakasugi & al. 1994) and parasitic plants (Wolfe & al. 1992; Haberhausen & Zetsche 1994). In green algae its presence was reported for *Nephroselmis olivacea* (Turmel & al. 1999), *Mesostigma viride* (Lemieux & al. 2000) and *Chaetosphaeridium globosum* (Turmel & al. 2002), but they were absent in *Chlorella* (Wakasugi & al.1997) and *Chlamydomonas reinhardtii* (Maul & al. 2002). The aim of this study was to investigate the presence of the plastid Ndh complex in chloroplasts of lichenized green algae. For this purposes we used *Ramalina farinacea*.

Materials and methods

Lichen material - *Ramalina farinacea* L. (Ach.) is a fruticose epiphytic lichen. The nature of the phycosimbiont is trebouxoid. This species, which occurs commonly in Mediterranean sclerophyllous oak forests, was collected in the air-dry state on *Quercus rotundifolia* Lam. at Sierra del Toro (south-western Castellón, 39° 54′16′′ N, 0° 48′ 22′′ W, Spain). On return to the laboratory, lichen thalli were transferred to a climatic chamber and maintained there for 3 days (200 mmol photon m^{-2} s^{-1}, 70% r.h., 18/14°C day/night 12-h photoperiod) to ensure full reactivation. The lichens were sprayed with distilled water once every morning to simulate the daily remoistening cycle.

Isolation of algal cells — Algal cells were isolated as described by Calatayud & al. (2001). *R. farinacea* thalli were homogenized in isotonic buffer (50 mM HEPES-NaOH pH 7.6; 0.33 M sorbitol, 2 mM EDTA), filtered through cheesecloth and centrifuged at 500xg. The pellets were resuspended in a minimal volume of isotonic buffer and loaded on a discontinuous density gradient formed by a lower layer of 80% PERCOLL® and an upper gradient of 50% PERCOLL.® After centrifugation at 30,000xg, isolated algae were recovered from the 50-80% PERCOLL® interface and washed twice in isotonic buffer. The PERCOLL® gradient procedure described by Calatayud & al. (2001) allows to isolate intact, physiologically active, algal cells. This method has the advantages to be simple and rapid. Algal preparations were essentially free of fungal contamination (less than 10.5% for *R. farinacea*). Purity of algal preparations is necessary for further detection of *ndh* genes products.

Gel electrophoresis — For the identification of *ndh* genes polypeptidic products, isolated algae were boiled in the presence of SDS, centrifuged to eliminate insoluble material and loaded on SDS-PAGE gels (O`Farrel 1975).

Immunoblot analysis - After electrophoresis, algal polypeptides were electrotransferred from gels to PVDF membranes. After incubation with the primary anti-NDH-F antibody, immunodetection was performed using a goat anti-rabbit Ig-G antiserum linked to horseradish peroxidase (Bio-Rad) as the second antibody. Color was developed using tetramethyl-bencidine and H_2O_2 as substrates. The anti-NDH-F antibody was previously developed against the NDH-F polypeptide of barley plastids (Catalá & al. 1997).

DNA isolation from algae - Algal cells were disrupted in a buffer containing 100 mM NaCl, 10 mM EDTA, 2% SDS, 100 mM TRIS-HCl pH 8.0. Phenol extraction of total algal DNA was performed at 70°C.

*PCR amplification and sequencing of the partial ndh*D *gene* - PCR amplification of a

*ndh*D fragment from total algal DNA was performed employing the primers 5'-CAT ACA TGG TTA CCA GAT AC-3' and 5'-TTC TGC AAC AAA TCC ACT CA-3'. The same primers were employed for sequencing of the purified amplification product. Nucleotide sequence was determined by the dideoxynucleotide chain-termination method (Sanger & al. 1977) with the ABI 377 system (Applied Biosystems). Sequence alignment was performed with Clustal W.

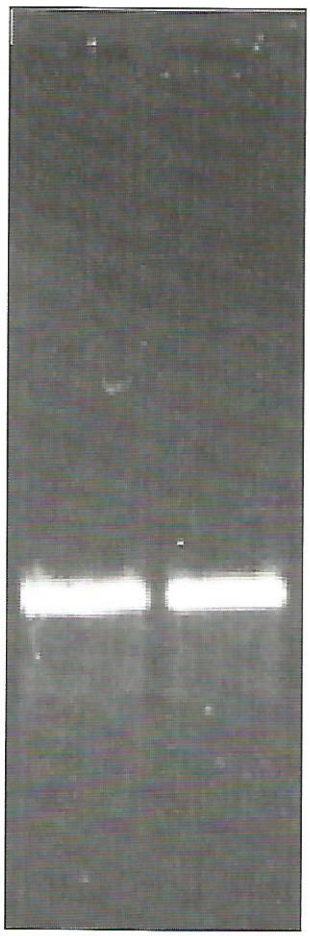

Fig. 1. PCR amplification of a partial *ndh*D gene sequence from *Ramalina farinacea* isolated photobionts DNA : left lane, Barley DNA; right lane, *Ramalina* DNA. The PCR conditions and the primers employed are as described in Materials and Methods.

Results and discussion

After isolation of total DNA from purified algae, presence of the gene encoding *ndh*D was first tested by PCR amplification using specific primers. We obtained a single PCR product of the expected size (approx. 500 bp) as shown in Fig. 1. This PCR product was

sequenced (Fig. 2). BLAST analysis clearly showed that this sequence corresponded to an *ndh*D gene. The sequence presented a high degree of similarity with the previously described *ndh*D sequence of *Physcomitrella patens* subsp. *patens* (Sugiura & al. 2003), a moss which is used as an alternative model system to investigate the molecular basis in land plants. The homology was lower when compared with *Anthoceros formosae* (Kugita & al. 2003), the Pteridophyte *Huperzia lucidula* (Wolf & al. 2003) or the unicelular green alga *Nephroselmis olivacea* (Turmel & al., 2002). These results clearly indicate the presence of the *ndh*D gene in Trebouxoid photobionts from *Ramalina farinacea*. They also suggest the possibility of a closer proximity of these *Trebouxia* plastid genome to the *Bryophytes* and/or the *Pteridophytes* included in the *Lycopodiales* -immediate ancestors of land plants- than to other green algae lineages.

Expression of the *ndh*F gene in lichenized algae from *Ramalina* was confirmed after western blotting (Fig. 3) of total protein extracted from lichen thalli (left lane), isolated algae (middle lane) and thylakoids from algal chloroplasts (right lane) using an anti-NDH-F specific antibody developed against the barley polypeptide. These results indicate that the presence of the Ndh complex has been conserved during the evolution of lichenized green algae. Therefore, a role increasing fitness should be assumed for this complex, at least under the lichenized state. Recent evidence indicates that the Ndh complex plays a

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R-farinacea       TATTTTGGGCATGTTATTTGCTGTTCCTCCCATGTGCTCAAGAAAAAGAGTGCGTGTTCT 60
P_patens          TATTTTAGGCATATAAATAGCTGTTCCTCCCATTTGATCTAAAAATAAAGTACGTGTTCG 60
A_formosae        AATTTTCGGTATAGAAGTAGCTATTCCTCCCATTTGATTAAGAAAAAGAGTACGTGTTCT 60
H_lucidula        TGTTTTAGGCATTGAAGTACCTATTCCTCCCATTTGGTCGAGGAAAGGGGTACGTGTCCT 60
M_polymorpha      TATTTTGGGCATAGAATTACCTATTCCACCCATTTGATCTAAAACCAAAGTCCGTGTTCG 60
P_nudum           GAGTCTTGGCATCGGAACAGCAATTCCTCCCAATTGATCAAGATAAAGAGTCTGTGTTCT 60
                     * * ** **   *    *  **** ****  ** *  *        **  **** *

R-farinacea       ATCATAACTTATTCCAGCTAAGAAAAAAAGTGCAGCACCAATTAATCCGTGAGAAATCAT 120
P_patens          ATCGTAAGTTATTCCAGCTAAGAAAAATAGTGCAGCACCAATTAATCCATGAGAAACCAT 120
A_formosae        ATCGTAAGTTACTCCTGCTGGAAAGAAGGGTGCAGCACCGATTAATCCATGAGAAATCAT 120
H_lucidula        ATCATAACTTGTTCCTGCCAGGGAGAATGGCGCAGCACCAATTAATCCGTGAGAAATCAT 120
M_polymorpha      ATCATAACTTATTCCTGCTAAGAAAAAAAGTGAAGCACCAATTAAACCATGTGAAATCAT 120
P_nudum           ATCGTAACTTATTCCTGCCAAAAAGAAAAGTGCTGCGCCTATTAATCCGTGGGAAATCAT 120
                  *** *** **  *** **     * **  * *  ** ** ***** ** ** **** ***

R-farinacea       TTGTAAAATAGCTCCATTAAAACCTAAATCGGTCATAGACCCAATGCCAACAAGTACAAA 180
P_patens          TTGTAAGATAGCGCCATTAAGACCTACATCTGTCATAGAACCAATACCAATAAGTACAAA 180
A_formosae        TTATGAAATAGCTCCATTAACACCTATATCTGTTGTAGGACCAATTCCGATAAGTACAAA 180
H_lucidula        TTGTAAAATAGCTCCATTTAAACCTATATTTGTAGCAGAACCAATTCCAATAAGTACAAA 180
M_polymorpha      TTGTAAAATAGCACCATTAAGCCCTAAATTTGTGATCGATCCAATTCCGATAAGAACAAA 180
P_nudum           TTGTAAAATAGCTCCATTTGTACCTATGTCTGTTATAGAACTAATTCCAATGATGACAAA 180
                  ** * * ***** *****    ****  *  **    *  * *** ** *  *  *****

R-farinacea       ACCCATACCGCGAAATTGTTGGGTAAGCAATTGTTGGTTTTAAA 224
P_patens          ACCCATAT-GTGATATTGAGGAATAAGCAATTCTTCGTTTTAAA 223
A_formosae        ACCCATGT-GAGAGACCAATAAATAAGCAATTCTTCTTTTTAAG 223
H_lucidula        ACCCATAT-GAGATATTGATGAATAAGCAATTCTTCTCTTCGAA 223
M_polymorpha      TCCCATAT-GTGATACTGAAGAATAAGCAATTCTTCTTTTTAAA 223
P_nudum           ACCCATAT-GCGAAACTGATGAATAAGCTATTCTTCTTTTGATA 223
                   *****   * ** *        ***** *** **   **
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Fig. 2. Partial sequence of the *ndh*D gene from *Ramalina farinacea* isolated photobionts DNA. The sequence is compared with the most similar from the Gene-Bank. Alignment of the sequences was performed using the program ClustalW.

role in the defense against photo-oxidative stress as produced by contaminant agents (Guéra & al. 2005). It is also well known that lichens can withstand sever water stress conditions, which produce a concomitant photo-oxidative stress, without loss of the photosynthetic capacity. Consequently, the conservation of a functional Ndh complex, in conjunction with the onset of the lichenization process, could contribute to the early colonization of land environments by some groups of green algae. Our results show for the first time the evidence that lichenized green algae contain *ndh* genes and that its polypeptide products are expressed.

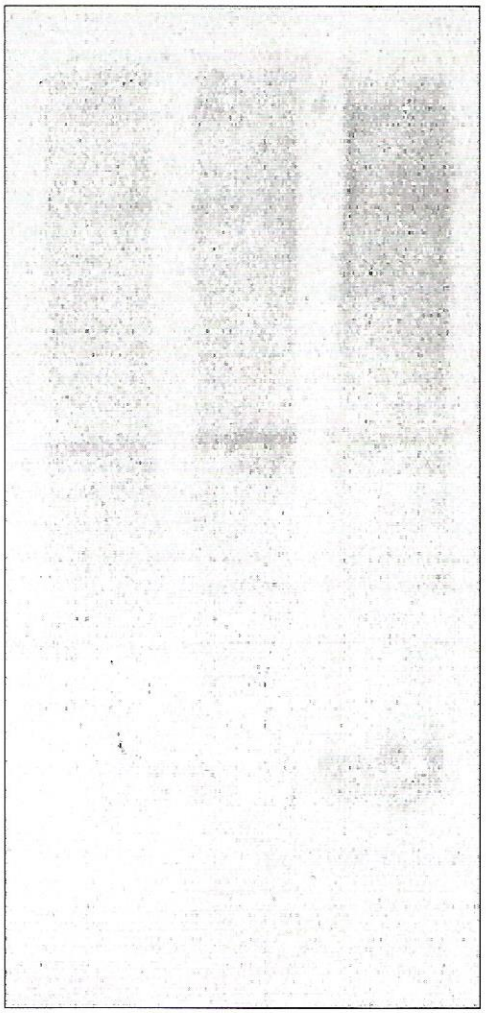

Fig. 3. Detection of the *ndh*F gene product in *Ramalina farinacea*: left lane, lichen extracts; middle lane, isolated algae; right lane, algal chloroplasts. The NDH-F polypeptide was detected after Western blotting by an anti-NDH-F antibody as described in Materials and Methods.

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