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RESEARCH PAPER

Chlorella saccharophila cytochrome *f* and its involvement in the heat shock response

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Abstract

Cytochrome *f* is an essential component of the major redox complex of the thylakoid membrane. Cloning and characterization are presented here of a novel partial cDNA (*ChspetA*) encoding cytochrome *f* in the psychrophile unicellular green alga *Chlorella saccharophila* and its involvement in the heat shock (HS) response pathway has been analysed. Semi-quantitative reverse transcriptase PCR analysis showed that *ChspetA* expression is up-regulated in heat-shocked cells and the protein profile of cytochrome *f* highlighted a release of cytochrome *f* into the cytosol depending on the time lapse from the HS. Evans Blue assay, analysis of chromatin condensation, and chloroplast alterations showed the induction of cell death in cell suspensions treated with cytosolic extracts from heat-shocked cells. This study identifies cytochrome *f* in *C. saccharophila* that seems to be involved in the HS-induced programmed cell death process. The data suggest that cytochrome *f* fulfils its role through a modulation of its transcription and translation levels, together with its intracellular localization. This work focuses on a possible role of cytochrome *f* into the programmed cell death-like process in a unicellular chlorophyte and suggests the existence of chloroplast-mediated programmed cell death machinery in an organism belonging to one of the primary lineages of photosynthetic eukaryotes.

Key words: Cell death, *Chlorella saccharophila*, chloroplast, cytochrome *f*, heat shock.

Introduction

Cytochrome *f* is one of the main subunits of an oligomeric membrane protein complex, the cytochrome b_6f, which is one of the major redox complexes of the thylakoid membrane. Cytochrome *f* is encoded by the single-copy chloroplast *petA* gene. The processed form of cytochrome *f* has a large NH_2-terminal heme binding domain anchored to the thylakoid membrane by a single 20-amino acid transmembrane helix. The heme group is bound to the protein by thioether bonds through conserved cysteine residues of a Cys-X-Y-Cys-His sequence found in all *c*-type cytochromes (Gray, 1992). Cytochrome *f* contains highly conserved basic residues which take part in the interaction with plastocyanin (Gong *et al.*, 2000). The three-dimensional structure of cytochrome *f* is unique among *c*-type cytochromes (Martinez *et al.*, 1994). The structure includes a rare buried chain of five water molecules in the heme-binding large domain (Martinez *et al.*, 1996) which is conserved throughout plant, cyanobacteria, and green algae cytochrome *f* (Martinez *et al.*, 1996; Carrell *et al.*, 1999). The cytochrome *f* protein sequence from green algae has been reported from *Chlamydomonas reinhardtii* (Chi *et al.*, 2000), *Chlorella vulgaris* (Wakasugi *et al.*, 1997), and from the psycrophile *Chlamydomonas raudensis* (Gudynaite-Savitch *et al.*, 2006).

Recently, a role for cytochrome *f* in programmed cell death (PCD) has been hypothesized in cultured eggplant cells (Peters and Chin, 2005). PCD is a genetically controlled process essential for development and stress response in plants and animals. It is characterized by certain morphological and biochemical features including the activation of caspases, cell shrinkage, chromatin condensation, and DNA fragmentation. Even though the events related to the PCD process have been well defined in animal cells, there are also increasing studies on PCD in plants and

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Abbreviations: HO, Hoechst 33342; HS, heat shock; PCD, programmed cell death; TEM, transmission electron microscopy.

recently a role for a PCD-like process in unicellular organisms has been recognized (Gordeeva *et al.*, 2004; Bidle and Bender, 2008). Moreover the absence of nitrogen in the culture medium and a reduced light induce PCD in the chlorophyte *D. terctiolecta* involving caspase-like proteases (Segovia *et al.*, 2003). Although some of the cellular responses in unicellular organisms resemble metazoan PCD, very little information is available for this process. It seems that, from an evolutionary point of view, across kingdoms there is some conservation in the PCD pathways (Berman-Frank *et al.*, 2004; Bidle and Falkowski, 2004; Moharikar *et al.*, 2006; Zuppini *et al.*, 2007). The finding of a self-destruction process triggered by environmental stresses in unicellular photosynthetic organisms similar to metazoan PCD suggests the existence of core PCD machinery in the early ancestor of plants and animals. Moreover, this process could have a widespread role in the control of cell survival, although the meaning of this process in a single-cell organism is still debated.

There has been much attention recently on the role of intracellular organelles in regulating PCD. The central regulatory role of mitochondria in integrating PCD signals in plant and animal PCD processes is well established (Apel and Hirt, 2004; Vacca *et al.*, 2004, 2006; Chalah and Khosravi-Far, 2008) as well as the participation of the endoplasmic reticulum in soybean stress-induced PCD (Zuppini *et al.*, 2004; Costa *et al.*, 2008). Moreover, it seems that, in both plants and animals, the cross-talk between the signalling cascades involving intracellular organelles in response to stress conditions is fundamental for the accomplishment of the PCD process (Sanges and Marigo, 2006; Zhang and Xing, 2008). Recently, a prominent role for chloroplasts in integrating PCD signals has been shown. Chloroplasts, in fact, are involved in plant and chlorophyte PCD pathways (Samuilov *et al.*, 2003; Zapata *et al.*, 2005; Yao and Greenberg, 2006; Zuppini *et al.*, 2007). It has been demonstrated that transgenic tobacco plants expressing anti-apoptotic genes coding for Bcl2-family proteins (Bcl-2, Bcl-xL and CED9) did not exhibit PCD characteristics when treated with chloroplast-targeted herbicides (Chen and Dickman, 2004). Moreover, it is well known that senescence is regulated by the chloroplasts (Zapata *et al.*, 2005). Recent findings highlight how several molecules related to the chloroplast (such as NADPH dehydrogenase, plastoquinone, AtMCP1b, and ACD2) are able to modulate PCD triggered by different abiotic and biotic stresses (Samuilov *et al.*, 2003; Li *et al.*, 2004; Castillo-Olamendi *et al.*, 2007; Yao and Greenberg, 2006). It has been demonstrated that a combination of reactive oxygen species and reduced plastoquinone of the photosynthetic electron transfer chain are necessary to initiate PCD in guard cells (Samuilov *et al.*, 2003) and that the chloroplast localized metacaspase AtMCP1b and ACD2 protein are involved in PCD triggered by pathogen infection (Castillo-Olamendi *et al.*, 2007; Yao and Greenberg, 2006). Cytochrome *f* is among the recognized proteins recognised to be involved in chloroplast-mediated PCD (Peters and Chin, 2005).

It has previously been shown that PCD occurs in the unicellular green alga *Chlorella saccharophila* in response to heat shock (HS). This PCD process has some of the characteristics of the metazoan PCD and seems to involve chloroplasts (Zuppini *et al.*, 2007). In this study, a release of cytochrome *f* from thylakoid membranes into the cytosol following the HS was detected in the psycrophile chlorophyte *C. saccharophila*. It was found that cytosolic extracts from treated cells, with elevated levels of cytochrome *f*, triggered cell death in a *C. saccharophila* cell population, suggesting a possible role for this protein in HS-induced PCD. Moreover, a partial *petA* cDNA from *C. saccharophila* (*ChspetA*) was isolated and identified and its expression analysed during HS-induced PCD. Our findings demonstrate a regulation of *ChspetA* transcripts and cytochrome *f* protein following the HS treatment. Overall, our analyses provide some insights into the PCD-like process in unicellular photosynthetic organisms, trying to dissect this intricate pathway which appeared early in evolution and has been maintained as an essential mechanism for the survival and development of all eukaryotic organisms investigated so far.

Materials and methods

Growth conditions and treatments

Chlorella saccharophila cells were grown in Bristol GR+ liquid medium (www.bio.utexas.edu/research/utex/) at a temperature of 4 °C and illuminated with white fluorescent tubes (800±100 lux) under a 16 h photoperiod. Cells were subcultured every 4 weeks, during the exponential growth phase, by making a 1:2 dilution in fresh medium. Cells in the exponential growth phase were incubated in a 44 °C prewarmed circulating water bath in the light for 2 h followed by incubation under normal growth conditions from 0 h to 72 h.

RNA preparation and cDNA synthesis

Control and treated cells were collected by centrifugation (5 min, 500 *g*, 4 °C) and frozen in liquid nitrogen. Total RNA was extracted with the 'RNeasy Plant Mini kit' (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. To eliminate any contamination with genomic DNA, the total RNA was treated with DNAseI (Promega, San Louis, CA, USA). The first strand cDNA was synthesized from 5 μg RNA using the 'Power Script Reverse Transcriptase' (BD Biosciences, San Jose, CA, USA) and 'Random Decamers' (Ambion, Austin, Texas, USA). The sample was diluted 1:5 and used for the polymerase chain reaction (PCR).

PCR

The *Chs*petA-FOR (5′-CCGGAAGGATTCGAATTAGCC-3′) and *Chs*petA-REV (5′-GCATCTCCTTGACCAAATC-CACCA-3′) primers were designed from conserved regions of known *petA* sequences from different organisms using the

'Primer3' software (http://frodo.wi.mit.edu/). PCR was carried out using 5 μl of diluted cDNA as a template and the 'Taq Advantage® 2' (Clontech, Mountain View, CA, USA) in a reaction volume of 50 μl. The amplification of the cDNA was performed according to the PCR program: 94 °C for 20 s, 54 °C for 30 s, 68 °C for 45 s for 35 cycles and a final extension at 70 °C for 10 min. The amplified products were analysed on 2% agarose gel stained with ethidium bromide. The PCR products were cloned into the 'pCR® 2.1-TOPO®' (Invitrogen, Carlsbad, CA, USA) and sequenced by 'BMR Genomics' (University of Padova). With this sequence information, new oligonucleotides were designed (*Chs*petA-C-Nter: 5′-TGGGACGATAACTCTGAAAAGA-3′ and *Chs*petA-D-Nter: 5′-TCCTTCCGGCAAAATAAGAACAGC-3′) and used in PCR reactions in combination with primers designed on the alignment of known *petA* 5′ (*Chs*petA-A-Nter: 5′-ACTCGCTCCATATCTGTCTCACTCAT-3′ and *Chs*petA-B-Nter: 5′-CGAGAGGCACTGGGCGTATT-3′) and 3′ (*Chs*petA-A-Cter: 5′-CTAGAAATTCATTTCGGACAATTGAAC-3′, *Chs*petA-B-Cter: 5′-GAACCTTCTCGAACTGTTTCTTTTTAAG-3′ and *Chs*petA-C-Cter: 5′-TTCTTTTTACAAACTTG-3′) regions. The resultant PCR products were cloned into the 'pCR® 2.1-TOPO®' (Invitrogen, Carlsbad, CA, USA) and sequenced by 'BMR Genomics' (University of Padova). The sequence of *Chs*petA was deposited in the GenBank database under Accession number FJ807388.

Semi-quantitative reverse transcription PCR

Semi-quantitative reverse transcription (RT)-PCR was carried out to verify the pattern of expression of *ChspetA* mRNA. Semi-quantitative RT-PCR was performed with 5 μl diluted first strand cDNA, using 18S rRNA as the internal standard ('Quantum RNA™ 18S internal standards', Ambion, Austin, Texas, USA). The oligonucleotide primers were designed from the *C. saccharophila petA* sequence obtained using the 'Primer3' software (http://frodo.wi.mit.edu/). The primers used were: *Chs*petA-I-FOR, 5′-TGGGAACAGAGGAAGGGGTCA-3′ and *Chs*petA-I-REV, 5′-CCACCACATTGGGATTGCTTG-3′. The amplification of the cDNA was performed according to the PCR program: 94 °C for 20 s, 61 °C for 30 s, 68 °C for 60 s for 35 cycles and a final extension at 70 °C for 10 min. Cycle numbers were optimized to ensure that the amplification reaction was tested in the exponential phase (data not shown). After purification with 'Microcon-PCR' (Millipore, Billerica, MA, USA) and sequencing, the PCR products obtained were resolved by electrophoresis on ethidium bromide-stained gels, scanned and quantified by using the 'Quantity One' and 'Molecular Analyst' software (Bio-Rad, Oakland, CA, USA). The quantification for *ChspetA* mRNA levels were normalized with respect to 320 bp of 18S rRNA.

Thylakoid membrane and cytosolic protein isolation

For thylakoid membrane isolation, control and treated cells were harvested by centrifugation (5 min, 500 *g*, 4 °C). Cell pellets (30 mg) were resuspended in buffer P1 (0.33 M sorbitol, 50 mM tricine pH 7.8, 5 mM $MgCl_2$, 10 mM NaCl, 1 μM leupeptin, and 0.5 mM phenylmethylsulphonyl fluoride) and homogenized with a 'Pellet pestle motor' (Sigma-Aldrich, St Louis, MO, USA) following five cycles of homogenization (30 s) and incubation in liquid nitrogen (1 min). Cell debris was pelletted by centrifugation at 4 °C (4 min, 2200 *g*) and the supernatant was centrifuged again for 30 min (18 000 *g*). The obtained pellet was homogenized in buffer P2 (50 mM tricine pH 7.8, 5 mM $MgCl_2$, 10 mM NaCl, 1 μM leupeptin, and 0.5 mM phenylmethylsulphonyl fluoride) and centrifuged for 10 min (18 000 *g*). The protein pellet enriched in thylakoid membranes was resuspended in buffer P3 (100 mM sorbitol, 50 mM tricine pH 7.8, 5 mM $MgCl_2$, 10 mM NaCl, 1 μM leupeptin, and 0.5 mM phenylmethylsulphonyl fluoride) and used for Western blot analysis. Cytosolic protein extracts were obtained as described by Estacion and Schilling (2002). Protein contents of the samples were determined by the method of Bradford (1976) using bovine serum albumin as a standard. The quality of the cytosolic protein extracts and the presence of contaminations by chloroplast proteins were verified by immunoblot analysis using a plant anti-D2 antibody (1:500, kindly provided by G Giacometti, Padova, Italy).

SDS-PAGE and immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli (1970). Membrane blotting was performed with the 'Semidry-system' (Bio-Rad, Oakland, CA, USA) according to the manufacturer's instructions. For protein identification 30 μg of proteins were subjected to 12% SDS-PAGE and transferred to polyvinyl difluoride (PVDF) membrane. Following transfer to PVDF, the proteins were visualized by staining with Ponceau Red to verify the protein loading and membranes were blocked with 5% (w/v) skimmed milk in phosphate saline (PBS) buffer for 1 h at room temperature and immunoblotted with a *Chlamydomonas reinhardtii* anti-cytochrome *f* antibody (1:5000, kindly provided by FA Wollmann, Paris, France) and a plant anti-D2 antibody (1:500, kindly provided by G Giacometti, Padova, Italy) for 2 h at room temperature. Membranes were then washed twice with 0.02% Tween/PBS followed by one wash in PBS before incubation in the secondary antibody. Labelling was detected with 'Sigma fast™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets' (Sigma-Aldrich, St Louis, MO, USA) and with chemiluminescence using 'CDP star™' (Biolabs, Hitchin, UK) and autoradiography films (Sigma-Aldrich, St Louis, MO, USA). The exposed films were quantified, after normalization with the protein content, by densitometric analysis using 'Quantity One' and 'Molecular Analyst' software (Bio-Rad, Oakland, CA, USA).

Evans Blue assay

The same amounts of cells in the exponential growth phase were incubated in 0.05% Evans Blue solution for 15 min

according to Levine *et al.* (1996). After extensive washing with deionized water, dye bound to dead cells was solubilized in 50% methanol/1% SDS for 30 min at 55 °C and quantified by absorbance at 600 nm. The evaluation of the percentage of dead cells in the population was carried out, considering as the 100% reference value the absorbance of an equal amount of cells treated for 10 min at 100 °C.

Fluorescent staining by Hoechst 33342

Control and treated cell suspension were stained with 8 μg ml^{-1} Hoechst 33342 (Sigma-Aldrich, St Louis, MO, USA) for 10 min at room temperature and analysed with a fluorescence microscope (excitation λ=350 nm). About 500 cells were analysed for each experiment.

Transmission electron microscopy

Cells were collected by centrifugation (5 min, 500 *g*, 4 °C) and fixed overnight at 4 °C in 3% glutaraldehyde in 0.1 M cacodylate buffer. Samples were dehydrated in a graded ethanol series and embedded in Epon resin. Ultrathin sections collected on copper grids were stained on-drop with uranyl acetate and observed with a Hitachi 300 electron microscope operating at 75 kV.

Statistical analysis

Data are presented as mean±SD of three independent experiments, using Student's *t* test to compare the significance of differences between data point ($P < 0.05$).

Results

Characterization and sequence alignment of a partial C. saccharophila petA *cDNA*

The *petA* is a chloroplast gene coding for cytochrome *f* protein. For *petA* cDNA cloning a first partial fragment of 490 bp was amplified by reverse transcription (RT)-PCR using total RNA isolated from *C. saccharophila* cells and primers designed from conserved areas of known *petA* genes (Fig. 1). The PCR product was cloned and sequenced showing a high similarity with known *petA* nucleotide sequences. In an attempt to find the full-length cDNA a nested approach was used. Two sets of primers were designed on conserved 5′ regions of known *petA* genes and 3′ *petA* untranslated regions (UTR) and used in combination with specific primers designed on the sequence of the amplified PCR product. A partial cDNA fragment was obtained encoding cytochrome *f* protein in *C. saccharophila*, called *ChspetA* (GenBank accession number FJ807388), consisting of 819 bp in length, missing about 150 bp of the 5′ end (Fig. 2).

Sequence analysis of *ChspetA* cDNA shows that it encodes for putative 275 amino acid residues (Fig. 2). The deduced amino acid sequence of *C. saccharophila petA* was aligned with known cytochrome *f* protein sequences with the ClustalW program (http://www.ebi.ac.uk/Tools). The result shows the highest similarity with plants (86–91%) and it reduces if the sequence is compared with *petA* from algae (69–77%) (Fig. 1). The deduced amino acid sequence includes the entire C-domain and part of the NH_2-terminal domain (Fig. 2). The *C. saccharophila* cytochrome *f* is composed of a NH_2-terminal domain and a highly conserved C-terminal domain. The ScanProsite program was used to analyse the protein profile of *Chs*petA. The analysis of the hits for Prosite motifs found the cytochrome *f* family profile (data not shown). The *C. saccharophila* apocytochrome *f* sequence comprises 28 negatively and 29 positively charged residues. The N-terminal protein sequence has the two conserved cysteine residues (CXXCH) located in the cytochrome centre for the heme binding (Fig. 2). Moreover, amino acid sequence analysis indicates the presence of residues involved in the formation of the 'water chain' and of a lysine residue involved in plastocyanin binding (Fig. 2). The presence of these typical residues is a key element for the identification and functionality of cytochrome *f* (Gudynaite-Savitch *et al.*, 2006). The deduced protein also exhibits the presence of the stromal extension and transmembrane anchor residues (Fig. 2). Notably, the stromal end of the helix (Leu-Val-Leu) is conserved (Fig. 2), similar to what was observed in some other algal species (Gudynaite-Savitch *et al.*, 2006).

Expression of ChspetA *in response to the heat shock treatment*

Total RNA isolated from *C. saccharophila* untreated and HS-treated cells, collected at different time point from the HS, was used to amplify *ChspetA* cDNA. Total RNA and specific primers designed on the previously obtained *ChspetA* sequence were used in PCR reactions. The ChspetA-I-FOR and ChspetA-I-REV primers were used to amplify a 200 bp region. The amplified RT-PCR fragment was cloned and sequenced, showing 100% identity with *ChspetA*. Semi-quantitative RT-PCR expression analysis showed that *ChspetA* cDNA has a constitutive transcript level in *C. saccharophila* control cells and was up-regulated 1 h after the HS (Fig. 3). For time intervals longer than 1 h (up to 72 h) the *ChspetA* transcript level is comparable to control cells (Fig. 3A). The 18S ribosomal RNA was used as the calibration standard as shown in Fig. 3A. A densitometry measurement showed that the induced level of *ChspetA* transcripts 1 h after the HS is about 1.3±0.02-fold higher than the constitutive level and the other HS treatments (Fig. 3B).

HS-induced cytochrome f *release and chloroplast alterations*

In a previous work by Zuppini *et al.* (2007), the involvement of chloroplasts in HS-induced programmed cell death has been postulated in *C. saccharophila* cells. The induction of cell death was related to the time elapsed from the treatment. In Fig. 4, protein extracts from control and HS-treated cells, enriched in thylakoid membranes and cytosolic

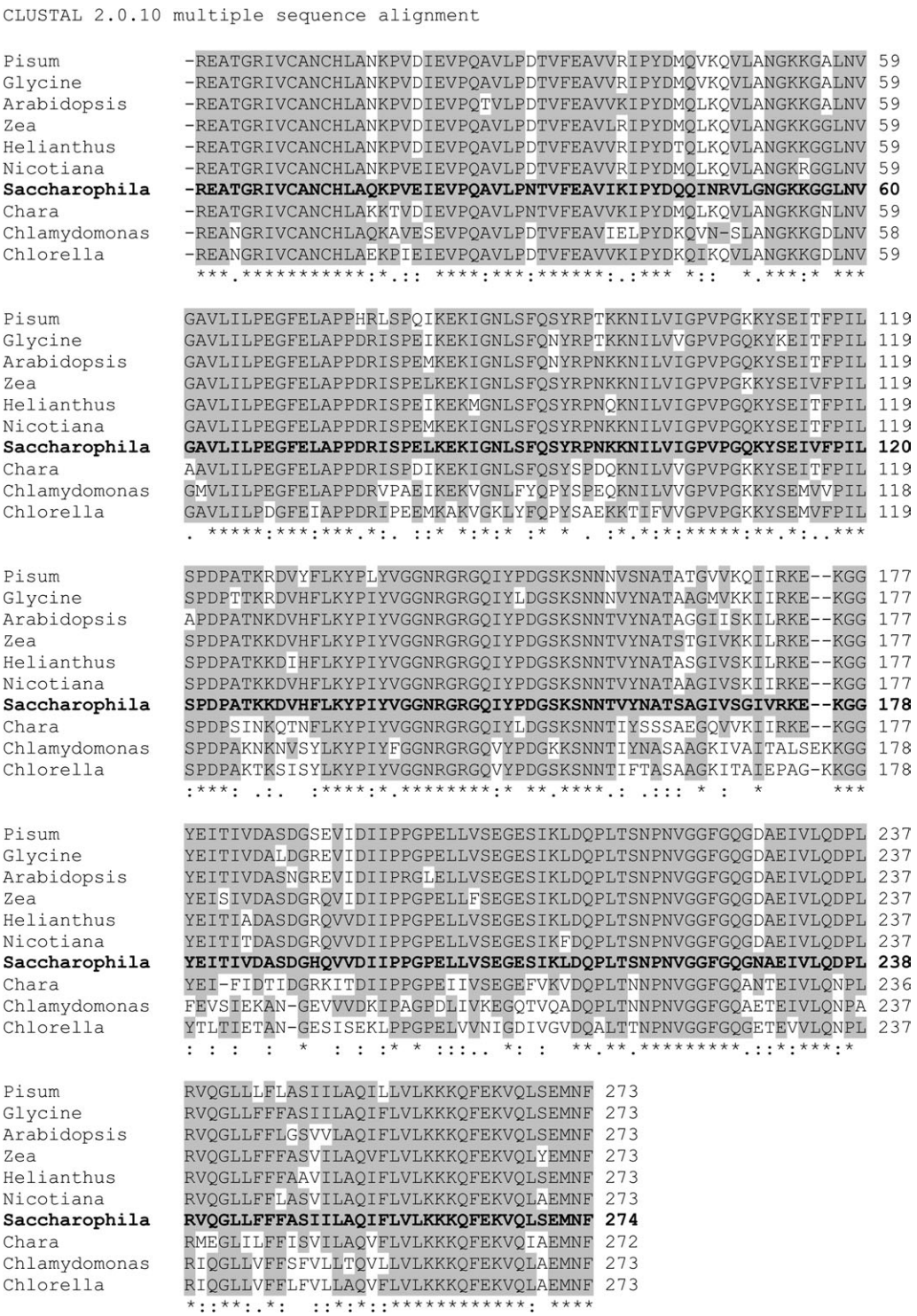

Fig. 1. Amino acid sequence alignment of cytochrome *f* protein from *Pisum sativum* (AAA85363), *Glycine max* (YP_538777), *Arabidopsis thaliana* (NP_051072), *Helianthus annuus* (YP_588130), *Nicotiana tabacum* (NP_054512), *Zea mays* (NP_043037), *Chlorella saccharophila* (this paper, FJ807388), *Chara vulgaris* (YP_635722), *Chlamydomonas reinhardtii* (CAA40911), and *Chlorella vulgaris* (NP_045911). The sequence of *C. saccharophila* cytochrome *f* was predicted from the DNA sequence obtained and aligned with the ClustalW program (http://www.ebi.ac.uk/Tools). Grey boxes indicate identical amino acids.

protein fractions, are analysed by Western blotting. The results indicate that a *C. reinhardtii* anti-cytochrome *f* antibody detects *C. saccharophila* cytochrome *f* as a single band of about 38 kDa. Control cells and cells treated with HS, analysed after 0–1 h from the HS, exhibit the presence of the protein band only in the enriched thylakoid membrane fractions (Fig. 4A), whereas 2 h after the HS the cytochrome *f* protein band is also detectable in the cytosolic fractions of the treated cells (Fig. 4C). From the protein profile analysis it seems that cytochrome *f* protein

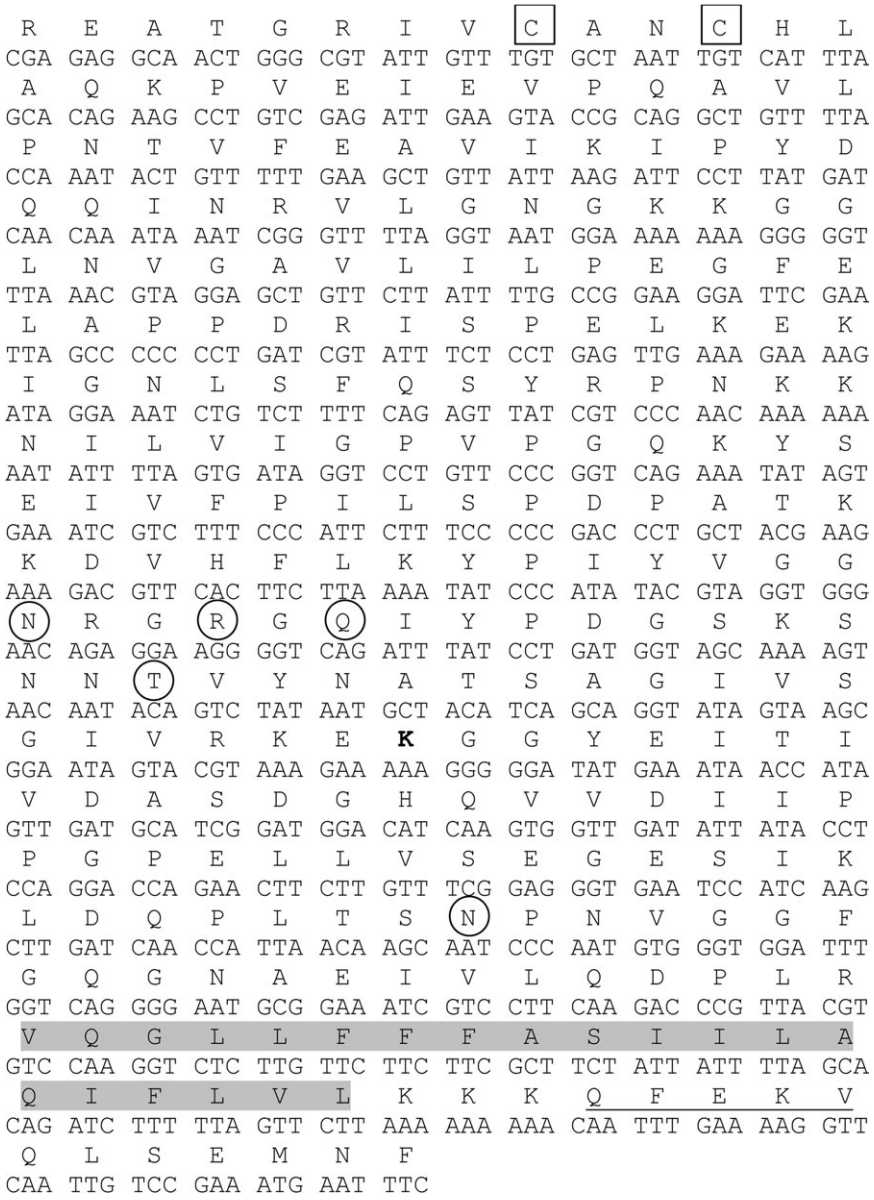

Fig. 2. *Chlorella saccharophila petA* cDNA and deduced amino acid sequences. The protein sequence was predicted from the DNA sequence using the ExPASy Translate Tool program (http://www.expasy.ch/tools/). Circles, amino acid residues involved in the water chain formation; bold, lysine residue involved in plastocyanin docking; highlighted, transmembrane helical domain; underline, stromal extension; boxes, heme binding.

expression is significantly induced within the first 2 h from the HS treatment (2.3±0.1-, 1.8±0.11-, and 1.6±0.12-fold for 30 min, 1 h and 2 h after the HS, respectively) (Fig. 4B) and that cytochrome *f* is progressively released in the cytosol after 2 h from the treatment (Fig. 4C, D). Immunoblot analysis of the D2 protein, an essential core component of photosystem II, provided indirect evidence on chloroplast envelope integrity. The protein, both in its phosphorylated and dephosphorylated form, was detected in the enriched thylakoid membrane fractions from the control and treated cells, whereas the reacting protein band was not present in the cytosolic fractions (Fig. 4E).

Transmission electron microscopy (TEM) was used to determine whether the release of cytochrome *f* in the cytosol was due to a HS-induced alteration of the chloroplast envelope. HS-treated cells were observed under TEM at the same time points as previously considered for cytochrome *f* release (0–2 h from the HS). Figure 5 shows that, immediately and after 30 min from the treatment, the most evident chloroplast structural alteration was the disorganization of the pyrenoid (Fig. 5F), when compared with the control (Fig. 5A). The pyrenoid, in control cells, is traversed by undulate and irregular single thylakoids and it is encircled by small osmiophilic pyrenoglobuli (Fig. 5A). In a short time after the HS the pyrenoid collapsed and the pyrenoglobuli are randomly distributed (Fig. 5C, F). Moreover, the organization of the thylakoid membranes changed: the thylakoids seemed to be less stacked with a swollen lumen (Fig. 5D, F). These ultrastructural features were also evident 2 h after the HS (Fig. 5G, H), while after

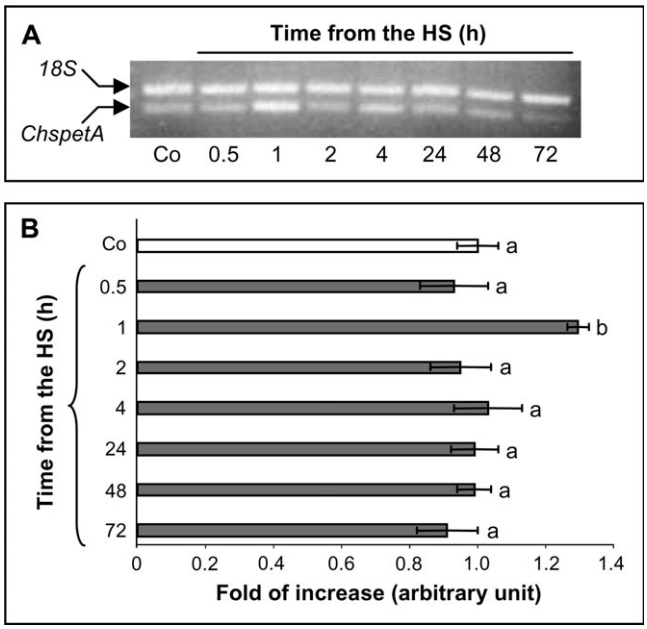

Fig. 3. Analysis of *ChspetA* gene expression. A volume of 5 μl PCR product was analysed using standard electrophoresis and observed using ethidium bromide (A). 18S rDNA (18S) accumulation was used as a constitutive expression control. Co, untreated cells. (B) Densitometry analysis of *ChspetA* expression in control (Co, white bar) and heat shock (HS)-treated cells (black bars). Relative transcript abundance was normalized against 18S rDNA. Data are mean±SD of three independent replicates. Bars labelled with different letters differ significantly (P <0.05) by Student's t test.

24 h the pyrenoid completely disappeared and the chloroplast internal membrane system was deeply affected (Fig. 5I). The chloroplast envelope, observed within 2 h from the treatment, did not show any evident structural alteration (Fig. 5D, F, G, arrows) and it is clearly unbroken as in the control cells (Fig. 5B, arrows). Only after 24 h had the chloroplast envelope been altered in its structure (Fig. 5I).

Cytochrome f *may be involved in the HS-induced cell death pathway*

With the aim of identifying a possible role for cytochrome *f* in the HS-induced cell death pathway, *C. saccharophila* cell cultures were treated with cytosolic protein extracts of *C. saccharophila* control cells and cells previously treated with a HS and collected at different time intervals from the HS. Based on the evidence that cytochrome *f* is detected in the cytosol 2 h after the HS, the *C. saccharophila* cell suspension was treated with cytosolic extracts of control, 2 h and 24 h HS-treated cells and the occurrence of cell death and the presence of chromatin condensation, one of the hallmarks of programmed cell death, were measured. The obtained results are shown in Fig. 6. Incubation of *C. saccharophila* cells for 24 h with cytosolic extracts from control cells (untreated) did not induce a significant increase in cell death compared with the control population (Fig. 6A), whereas 2 h and 24 h cytosolic extracts induced a significant increase in cell death (2.2±0.1-fold and 3.0±0.3-fold, respectively), compared with the control (Fig. 6A). Moreover, it was possible to detect the presence of chromatin condensation in cells treated with the 2 h and 24 h cytosolic extracts (Fig. 6B). Hoechst positive nuclei (blue staining) were detected in cells treated with both 2 h and 24 h cytosolic extracts. An increase in the number of stained cells from 5–6% in the control and cells treated with cytosolic control extracts to about 20–21% of cells treated with cytosolic extracts from heat-shocked cells has been observed (Fig. 6B).

Discussion

In this work, a new partial *petA* cDNA was identified from *C. saccharophila* (*ChspetA*) coding for cytochrome *f*, an integral thylakoid membrane protein. Its expression and release in the cytosol is correlated with HS-induced PCD. Cytochrome *f* is encoded by the cloroplastic gene *petA* and thus the protein is not imported from the cytosol. The sequence of *ChspetA* cDNA revealed that it consists of 819 bp. By a search of the cytochrome *f* sequences from the GenBank database and multiple sequence alignments the deduced *C. saccharophila* cytochrome *f* amino acid sequence has the highest percentage of similarity with plants (about 90%) and *Chara vulgaris* (77%), whereas the similarity reduces to 67–69% if the *C. saccharophila* cytochrome *f* amino acid sequence is compared with green algae. Multiple alignments of the cytochrome *f* amino acid sequences revealed that the C-terminal domain is highly conserved in *C. saccharophila*. The conservation of the C-domain amongst plants and algae cytochrome *f* is high, especially with regard to the stromal extension of the protein, whereas the transmembrane helical domain is less conserved throughout evolution (Choquet *et al.*, 2003). The *C. saccharophila* C-domain has conserved both the stromal extension and the transmembrane helix; in particular, the stromal end of the helix in *C. saccharophila* cytochrome *f* has the same amino acid sequence (Leu-Val-Leu) found in other algal species (Choquet *et al.*, 2003). Moreover, the residues involved in the formation of the water chain are present in *C. saccharophila* cytochrome *f*. Cytochrome *f*, in fact, has an internal network of water molecules coordinated by highly conserved residues (Sainz *et al.*, 2000). This finding confirms the phylogenetic conservation of these residues throughout the photosynthetic organisms. This rare, buried water chain has been found in few molecules and the high conservation of residues in cytochrome *f* is suggestive of a critical function. It has been hypothesized that this chain functions as a proton wire and is essential for photosynthetic function (Martinez *et al.*, 1996; Sainz *et al.*, 2000). It has been postulated that cytochrome *f* translation is self-regulated via some motives that extend in the short stromal stretch of the C-terminal part of the protein (Choquet *et al.*, 2003). It seems that cytochrome *f* synthesis is mediated by the 5′-UTR of the *petA* mRNA. In the sequence of *C. saccharophila* cytochrome *f*, the four

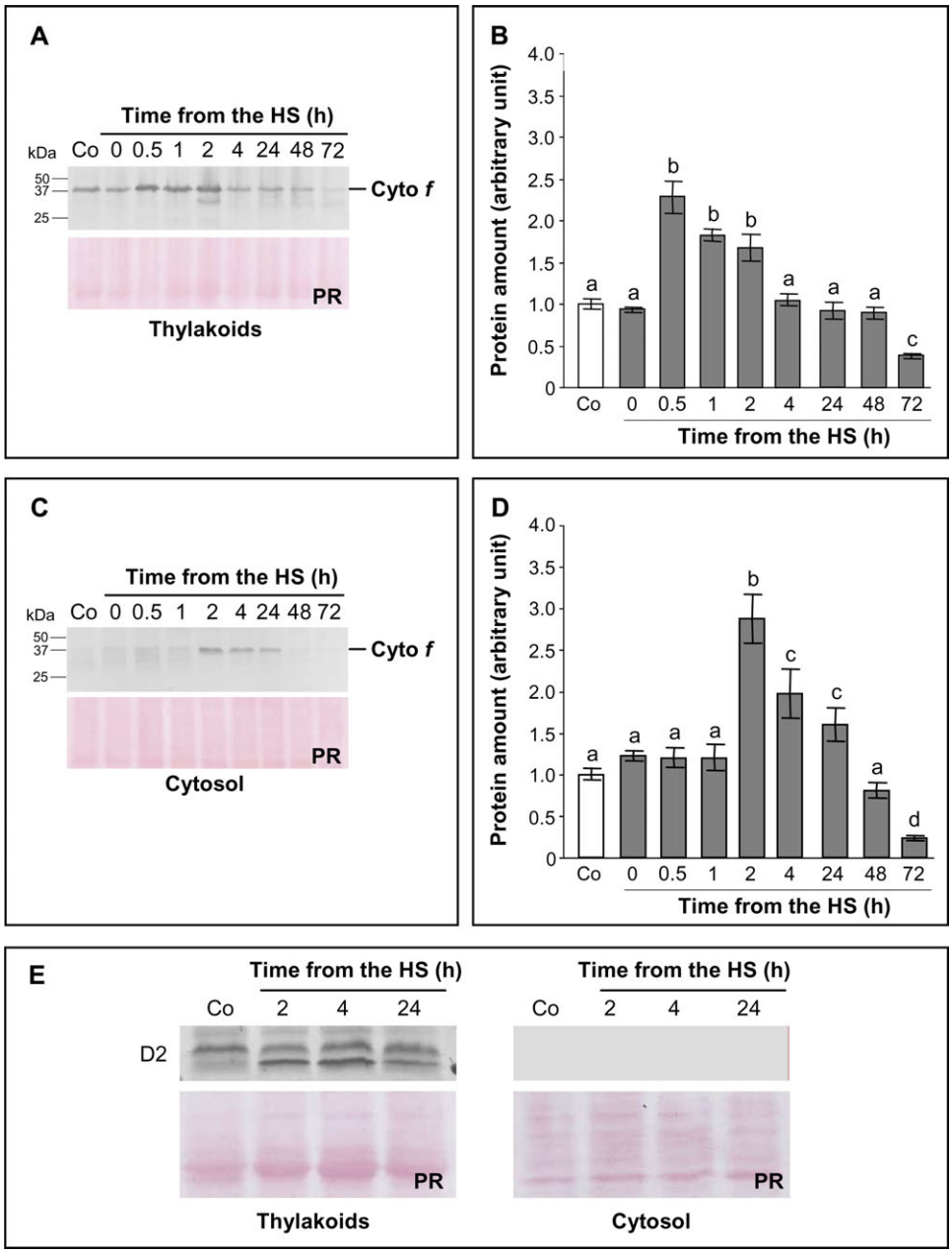

Fig. 4. Changes in cytochrome *f* localization and D2 protein following heat shock (HS) treatment. Presence of cross-reacting cytochrome *f* (Cyto *f*) and D2 protein band was assessed by immunoblot analysis using Cyto *f* and D2 specific antibodies (A, C, E). Relative protein amount of control (Co, white bar) and HS-treated cells (grey bars) was normalized against protein loading and analysed by densitometry analysis of the protein band (B, D). Data are mean±SD of three independent replicates. Bars labelled with different letters differ significantly (P <0.05) by Student's t test. Thylakoids, protein fractions enriched in thylakoid membranes; Cytosol, cytosolic protein fractions; PR, Ponceau red staining; Co, untreated cells.

positively charged Lys residues (position 303 to 305 and 309), probably involved in the electrostatic interaction with the *petA* gene 5′-UTR, are present. These findings suggest that *C. saccharophila* cytochrome *f* shares conserved amino acids and cytochrome *f* domain signatures, highlighting the maintenance of its cellular roles.

There is increasing evidence that common ancestral apoptotic machinery exists among eukaryotes (Bidle and Falkowski, 2004; Gordeeva *et al.*, 2004). However, very few molecular components involved in programmed cell death (PCD) of plants and unicellular organisms have been determined. The occurrence of PCD in *C. saccharophila* following a heat treatment has been reported previously (Zuppini *et al.*, 2007). In particular, *C. saccharophila* cells seem to undergo PCD via a caspase-mediated pathway involving the chloroplasts; alterations in chloroplast morphology and protein and pigment contents have been detected (Zuppini *et al.*, 2007). In this work, *C. saccharophila* was used as a model system to identify molecules involved in the HS-induced PCD process.

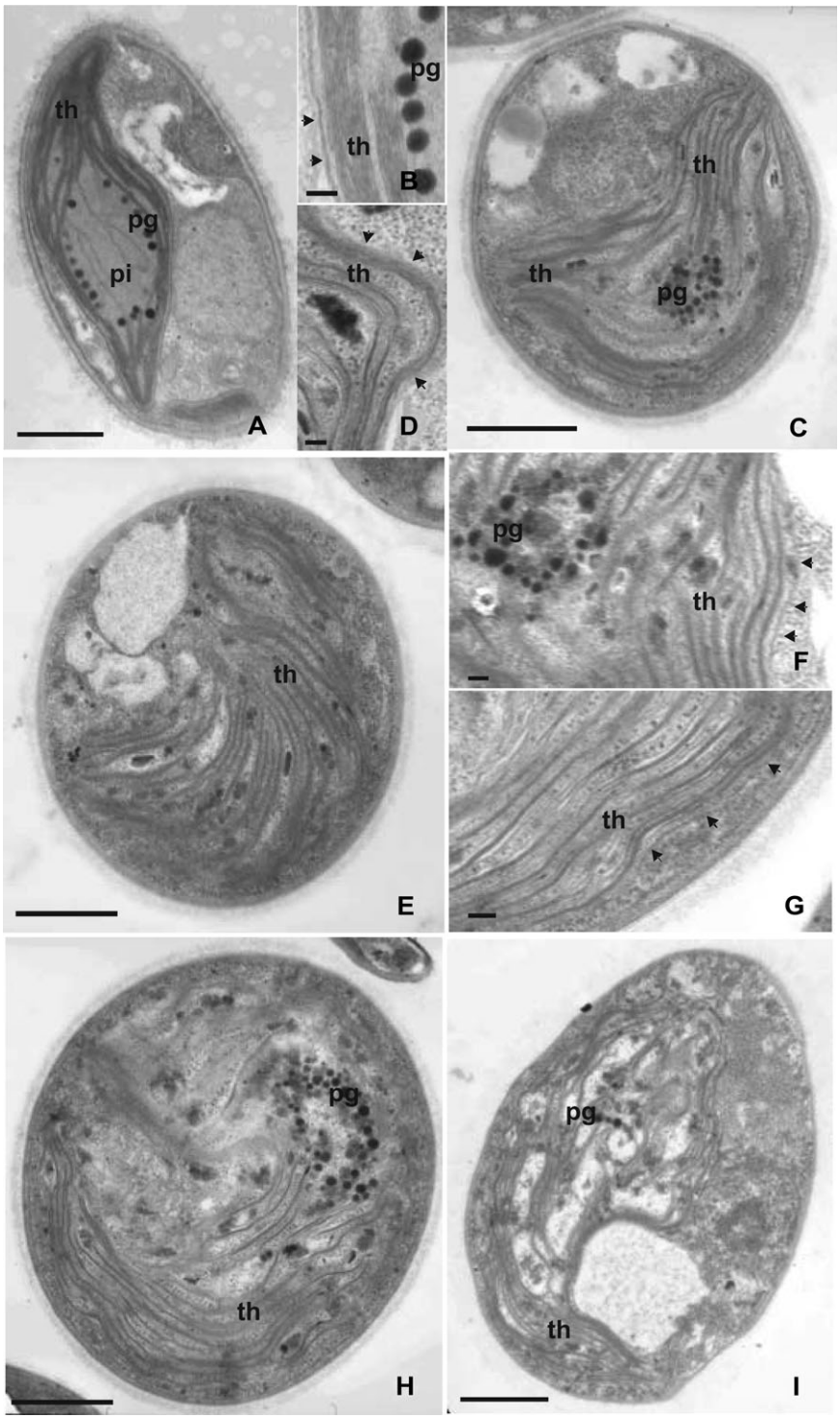

Fig. 5. Effect of the heat shock (HS) treatments on chloroplast ultrastructure of *C. saccharophila* cells. (A, B) Control cells; (C, D) HS-treated cells; (E, F) HS-treated cells analysed 0.5 h after the HS; (G, H) HS-treated cells analysed 2 h after the HS; (I) HS-treated cells analysed 24 h after the HS. Arrows indicate the chloroplast envelope. pg, Pyrenoglobuli; pi, pyrenoid; th, thylakoids; bar: 1 μm (A, C, E, H, I); 0.1 μm (B, D, F, G).

In the last few years, the role of chloroplasts as key organelles in the execution of PCD in plant cells is emerging (Samuilov *et al.*, 2003; Zapata *et al.*, 2005; Yao and Greenberg, 2006; Zuppini *et al.*, 2007). A number of molecules directly or indirectly related to the chloroplast seem to be crucial in the execution of some PCD pathways (Peters and Chin, 2005; Yao and Greenberg, 2006). In the hypersensitive response and after pathogen infection, chloroplastic factors seem to be implicated in the pathway leading to cell death (Karpinski *et al.*, 2003; Castillo-Olamendi *et al.*, 2007) and the ectopic expression in the chloroplasts of anti-apoptotic Bcl-2 proteins protects

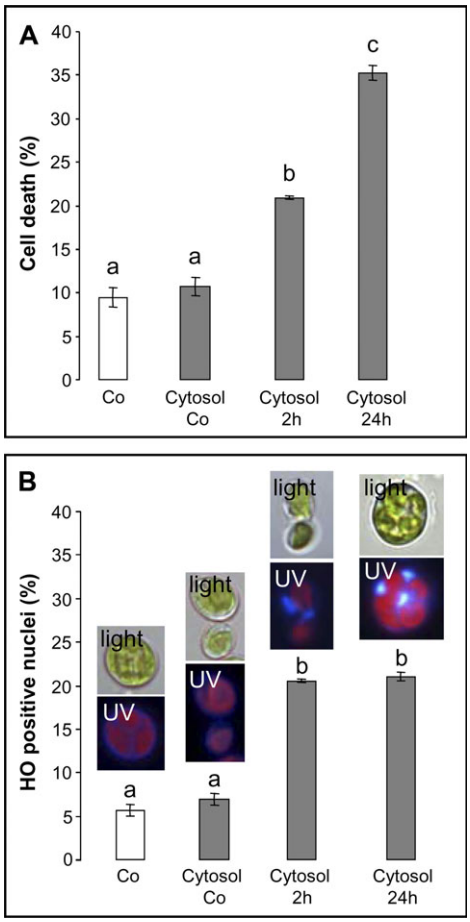

Fig. 6. Cell viability and chromatin condensation in cells treated with cytosolic extracts. Exponentially growing cells were incubated with cytosolic extracts of control (cytosol Co) and heat shock-treated cells (cytosol 2 h and 24 h). (A) The 100% value corresponds to cells treated for 10 min at 100 °C. (B) Cells were stained with Hoechst 33342 (HO) and observed under the fluorescence microscope (blue staining). Pictures represent typical examples. Data are mean±SD of three independent experiments. Bars labelled with different letters differ significantly (P <0.05) by Student's t test. Co, untreated cells.

tobacco plants from herbicide-induced PCD (Chen and Dickman, 2004). Moreover, Peters and Chin (2005) demonstrated that treatment of eggplant cells with palmitoleic acid induced cell death via a release of cytochrome *f* in the cytosol, highlighting the role of this protein in cell death.

An initial characterization of the possible role of cytochrome *f* during HS-induced PCD was attempted. *C. saccharophila* cells were treated with a 44 °C temperature for 2 h and analysed at different times after the HS. In a previous work the occurrence of cell death following a HS has been demonstrated and it seems that this PCD pathway leads to alterations in chloroplast morphology and protein contents (Zuppini *et al.*, 2007). A comparison of *ChspetA* mRNA expression in control and treated cells showed that the gene is induced early after the HS. The results suggest the possibility that *ChspetA* expression is induced by HS treatment. Moreover, whilst analysing cytochrome *f* protein levels in thylakoid membrane and cytosolic fractions, an increase in the level of the protein in the thylakoid membranes and a release of cytochrome *f* in the cytosol was detected 2 h after the HS. Thus, protein synthesis seems to be induced by the HS treatment and the protein is probably inserted into the thylakoid membrane to be released in the cytosol afterwards. TEM analysis highlighted an alteration of the thylakoid membrane structure whereas the integrity of the chloroplast envelope is not affected by the HS treatment. Intriguingly, analysing the location of an integral thylakoid membrane protein of photosystem II, the D2, no reacting protein band could be detected in the cytosolic fractions. These data strengthen the hypothesis that cytochrome *f* release and gene expression are correlated with the HS-response. If this, in fact, was due to a non-specific dismantling of the thylakoid membranes we should have found the D2 protein in the cytosolic fractions.

Chloroplasts, together with mitochondria, are the prime sites of reactive oxygen species production and they may generate intermediate signals involved in PCD (Apel and Hirt, 2004; Van Breusegem and Dat, 2006). The functional parallelisms between chloroplast and mitochondria, together with increasing evidence on the key role of the chloroplast in plant PCD lead to hypothesize a role for chloroplast-derived signals in plant PCD similarly to what is found for mitochondria-mediated PCD. It seems that chlorophyll catabolism products are involved in PCD during plant cell infection with *P. syringae* (Yao and Greenberg, 2006). Moreover, several proteins related to the chloroplast, such as ACD1, ACD2, and atMCP1b, are involved in different ways in the PCD process (Tanaka *et al.*, 2003; Watanabe and Lam, 2005; Yao and Greenberg, 2006). The release of apoptogenic protein from the mitochondrial inner space, such as cytochrome *c*, triggers apoptosis in mammalian and plant cells (Vacca *et al.*, 2006; Chalah and Khosravi-Far, 2008). It has been demonstrated that, in eggplant cells, cell death induced by palmitoleic acid occurs with a release of cytochrome *f* in the cytosol (Peters and Chin, 2005). The meaning and the mode of action of cytochrome *f* in the PCD pathway remain to be elucidated: it is not clear if the involvement of cytochrome *f* in the PCD process is related to its loss of function or if the protein by itself is an important regulatory factor in HS-induced PCD. Interestingly, treatment of the *C. saccharophila* cell suspension with protein extracts from HS-treated cells, containing cytochrome *f*, induces cell death occurring with chromatin condensation. Thus, some elicitor(s) in these extracts induces death in cells. Based on previous data on the occurrence of cell death correlated with an accumulation of cytochrome *f* in the cytosol (Peters and Chin, 2005), we are confident that this protein could also have a similar role in *C. saccharophila*. Further experiments will be addressed in this direction.

Due to the important findings on the ancestral origin of the PCD machinery, it is extremely interesting trying to define the common components of the cell death pathways throughout evolution. From an evolutionary point of view

C. saccharophila belongs to one of the primary lineages of photosynthetic eukaryotes (Delwiche, 1999). Thus, it seems that chloroplast-mediated PCD machinery is also present in green algae and that the chloroplast could also have a central regulatory role in integrating stress and/or PCD signals in unicellular organisms. Moreover, the PCD process has been demonstrated to occur in photosynthetic and non-photosynthetic prokaryotes as well (Ning *et al.*, 2002; Bidle and Falkowski, 2004): it is rational to suppose, considering the endosymbiontic origin of mitochondria and chloroplasts, that these organelles could have a role in PCD involving some of their own molecules. Overall, our results focus on the possible role of cytochrome *f* as an important factor in the pathway of cell death induced by HS in unicellular algae. At present, there is increasing interest in the role of the chloroplast in plant PCD because chloroplast modifications and a cross-talk between chloroplast and mitochondria seem to be involved in triggering PCD in response to stress (Zhang and Xing, 2008). The result presented in this work, together with our previous findings on HS-induced PCD, represents an interesting starting point to understand the origin and the evolutionary meaning of the chloroplast-mediated PCD machinery.

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