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Cloning, expression and purification of cytochrome c_6 from the brown alga *Hizikia fusiformis* and complete X-ray diffraction analysis of the structure

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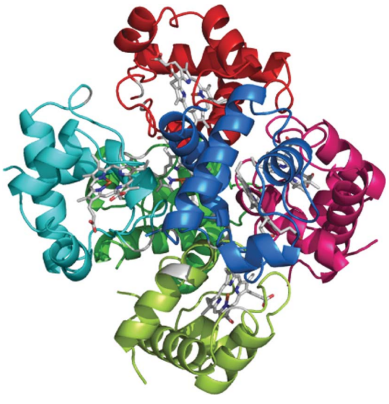

The primary sequence of cytochrome c_6 from the brown alga *Hizikia fusiformis* has been determined by cDNA cloning and the crystal structure has been solved at 1.6 Å resolution. The crystal belonged to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 84.58$, $c = 232.91$ Å and six molecules per asymmetric unit. The genome code, amino-acid sequence and crystal structure of *H. fusiformis* cytochrome c_6 were most similar to those of red algal cytochrome c_6. These results support the hypothesis that brown algae acquired their chloroplasts *via* secondary endosymbiosis involving a red algal endosymbiont and a eukaryote host.

1. Introduction

Soluble *c*-type monohaem cytochromes are ubiquitously distributed haem proteins which act as electron carriers in mitochondria, bacteria, algal chloroplasts and cyanobacteria. Cytochrome c_6 is a soluble low-spin haem protein that functions in oxygenic photosynthesis as an electron carrier between cytochrome *f*, which is part of the membrane-embedded cytochrome b_6f complex, and the P700 reaction centre of photosystem I (Kerfeld & Krogmann, 1998). This cytochrome c_6 is classified as a class I *c*-type cytochrome, in which the haem iron has histidine–methionine axial coordination. Plastocyanin is a blue copper protein with the same function as cytochrome c_6. Cytochrome c_6 and plastocyanin have completely different amino-acid sequences and secondary and tertiary structures, but they contain similar acidic and hydrophobic patches on their surface for recognition of their interaction partners (Frazão *et al.*, 1995; Ullmann *et al.*, 1997).

Although chloroplasts are thought to have evolutionally arisen from cyanobacteria (Aitken, 1976), there are differences in the expression and genome coding of cytochrome c_6 in green and red algae. In the green alga *Chlamydomonas reinhardtii*, the gene for cytochrome c_6 exists in the genomic DNA and its coding region is interrupted by two introns (Hill *et al.*, 1991). On the other hand, in the red alga *Porphyra purpurea* the *petJ* gene encoding cytochrome c_6 exists in the chloroplast genome (Reith & Munholland, 1993).

In eukaryotic brown algae, which contain no plastocyanin, photosynthetic electron transport between cytochrome *f* and photosystem I is only performed by cytochrome c_6. It is generally considered that brown algae acquired their chloroplasts *via* secondary endosymbiosis involving a primitive red algal endosymbiont and a nonphotosynthetic eukaryote host (Cavalier-Smith, 2000; McFadden, 1999). Although the physicochemical properties and amino-acid sequences of cytochromes c_6 from the brown algae *Petalonia fascia* and *Alaria esculenta* have been determined (Sugimura *et al.*, 1981; Laycock, 1975), the genome code and tertiary structure of brown algal cytochrome c_6 remain to be studied. In this study, we determined the genome code of the brown algal cytochrome c_6 gene from the brown alga *Hizikia fusiformis*, determined the crystal structure of the protein and compared it with those of cyanobacterial and red and green algal cytochromes c_6.

2. Materials and methods

2.1. Sequence determination

The brown alga *H. fusiformis* was collected in the coastal area off Hayama, Japan. Total RNA was isolated from the brown alga using the RNeasy Plant Mini Kit (Qiagen). Poly(A)$^+$ mRNA and poly(A)$^-$ mRNA were separated from the total RNA using Oligotex-dT30 (Takara). Additional of adenine at the 3′-terminus of poly(A)$^-$ mRNA was carried out for 40 min at 310 K in a reaction mixture containing 2 µg poly(A)$^-$ mRNA, 50 m*M* Tris–HCl pH 7.9, 50 m*M* $MgCl_2$, 10 m*M* $MnCl_2$, 500 m*M* NaCl, 2.5 m*M* DTT, 0.5% BSA, 1 m*M* ATP, 121 U ribonuclease inhibitor and 1.5 U poly(A) polymerase (Takara). First-strand cDNA was synthesized using a 1st Strand cDNA Synthesis Kit with AMV Reverse Transcriptase (Life Science Inc.) and the oligonucleotide primer 5′-CGGGATCC$(T)_{25}$-3′, designated primer P1 (reverse). To obtain the clone encoding the 3′-region of cytochrome c_6 from *H. fusiformis*, we designed the degenerate oligonucleotide primer P2 (forward), 5′-AAYTGYGCIGCIGCIT-GYCAYGCI-3′, based on the highly conserved residues around the haem *c* motif (Asn-Cys-Ala-Ala-Cys-His-Ala) of cytochrome c_6 from the cyanobacteria *Synechocystis* PCC6803 and *Anabaena* 7119, the green alga *C. reinhardtii*, the euglena *Euglena gracillis* and the cyanelle *Cyanophora paradoxa*. PCR products were subcloned into a pGEM T-Easy vector (Promega). DNA sequencing was performed by the dideoxy chain-termination method using a Thermo Sequence fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amasham) and an automated DSQ 2000L DNA sequencer (Shimadzu, Japan). The first-strand cDNA from *H. fusiformis* were dC-tailed at their 3′-ends using the 5′ RACE system for Rapid Amplification of cDNA Ends Reagent Assembly v.2.0 (Life Technologies Inc.). The 5′-region of the cytochrome c_6 gene from *H. fusiformis* was amplified by the polymerase chain reaction (PCR) using a forward primer complementary to the dC tail [P3, 5′-GGCCACGCGTCGACTAGTAC$(G)_{16}$-3′] and a gene-specific primer designed based on the 3′-region sequences of the cytochrome c_6 cDNA (Hf1, 5′-TCAGGCATAATAATAACATTATTACCGCC-3′). The PCR product was subcloned and sequenced by the same methods as used in 3′ RACE. Genome DNA from *H. fusiformis* was extracted using Isoplant II (Nippon Gene). To obtain the genome sequence of *H. fusiformis* cytochrome c_6, we designed gene-specific primers for amplification of the full-length cytochrome c_6 gene on the basis of the cDNA sequence of cytochrome c_6 (Hf2, 5′-ATGGGGGGGTGGAAAATTTATTATT-3′, forward; Hf3, 5′-TCAACGTTCCAGGTCCAATAATATCATAA-3′, reverse). The PCR product was subcloned and sequenced according to 3′ RACE.

2.2. Construction of expression vector

Construction and overproduction of the cytochrome c_6 gene (*petJ*) in *Escherichia coli* was performed according to the method described by Satoh *et al.* (2002) with slight modifications. The mature cytochrome c_6 sequence was amplified using the forward primer ExP1 (5′-CATGCCATGGGCTGATATTAATCATGGAG-3′) corresponding to codons for the amino-acid residues of the cytochrome c_6 N-terminal region and the reverse primer ExP2 (5′-GCGGATCCTTAGT-TCCAACTCTTTTCAG-3′) corresponding to codons for the amino-acid residues of the C-terminal region. The amplified mature cytochrome c_6 sequence was ligated to the *pelB* signal sequence adapter (Genset Co. Ltd). The resulting *pel*B–cytochrome c_6 hybrid gene was cloned into *Nde*I–*Bam*HI sites of pET22b(+) (Novagen Co. Ltd) to construct the plasmid pET22bHfc6.

The cytochrome *c* maturation genes *ccmA–H* were amplified using the polymerase chain reaction from *E. coli* MC1061 genomic DNA using the forward primer P3, 5′-CCAGAATTCGGTTGCCGC-GAAGATGCAT-3′, corresponding to upstream of the *ccmA* gene from the *E. coli* K12 MG1655 genome sequence (AE000309), and the reverse primer P4, 5′-TTCCTGCAGCAACGCGGGGCACAATA-AA-3′, corresponding to downstream of the *ccmH* gene. The resulting *ccmA–H* gene was cloned into the *Eco*RI–*Pst*I sites of pSTV28 (Takara Shuzo Co.) to create the plasmid pSTV28*ccmA–H*.

2.3. Protein expression and purification

For the overproduction of *H. fusiformis* cytochrome c_6, both pET22bHfc6 and pSTV28*ccmA–H* were co-introduced into *E. coli* BL21 (DE3). Transformed *E. coli* cells were grown in 1 l Luria–Bertani (LB) medium supplemented with 100 mg l^{-1} ampicillin and 20 mg l^{-1} chloramphenicol at 303 K for 36 h. Cells were harvested by centrifugation at 6000*g* (277 K) for 5 min. The pellet was resuspended in 80 ml PBS buffer and disrupted using a high-pressure homogenizer (Mini Lab 8.30H, Rannie). The suspension was fractionated with ammonium sulfate (40–80% saturation). The precipitate was dissolved in a small amount of 20 m*M* sodium acetate buffer pH 5.5 and dialyzed against the same buffer. The sample was applied onto a DE52 cellulose column (Whatman, 2.0 × 40.0 cm) equilibrated with 20 m*M* sodium acetate buffer pH 5.5. After the column had been washed with the same buffer, the proteins were eluted using a linear gradient of sodium acetate pH 5.5 (20–200 m*M*). Fractions containing cytochrome c_6 were pooled and dialyzed against 20 m*M* sodium acetate buffer pH 5.5 and the dialyzed sample was applied onto a Poros HQ/20 column (Applied Biosystems) previously equilibrated with the same buffer. After the column had been washed with the same buffer, the proteins were eluted with an NaCl gradient (0–500 m*M*) in the same buffer. The sample thus obtained was used as purified recombinant *H. fusiformis* cytochrome c_6. The degree of purity was confirmed by tricine SDS–PAGE (Schägger & von Jagow, 1987) and UV–visible spectroscopy. UV–visible spectra of *H. fusiformis* cytochrome c_6 were measured with a Hitachi U3310 spectrophotometer using quartz cuvettes of 1.0 cm path length. The concentration of the cytochrome c_6 was determined spectrophotometrically from the pyridine ferrohaemochrome spectrum (550 nm, 29.1 mM^{-1} cm^{-1}). Potassium ferricyanide and sodium dithionite were used as the oxidant and the reductant, respectively.

2.4. Crystallization and refinement

The purified protein was dissolved in 10 m*M* sodium phosphate buffer pH 7.0 to prepare a concentrated protein solution of 20 mg ml^{-1}. Initial crystals were obtained using the Wizard I random sparse-matrix crystallization screen (Emerald BioSystem). *H. fusiformis* cytochrome c_6 was crystallized by vapour diffusion using the hanging-drop method at 293 K. Each drop consisted of 2 µl protein solution and 2 µl reservoir solution. An initial crystal of *H. fusiformis* cytochrome c_6 grew within a week using condition No. 33 [2.0 *M* $(NH_4)_2SO_4$, 0.1 *M* CAPS pH 10.5 and 0.2 *M* Li_2SO_4]. To improve the quality of the crystal, further screening for crystallization was performed and crystals were obtained reproducibly using 0.1 *M* CAPS pH 10.5, 0.2 *M* Li_2SO_4, 2.2 *M* $(NH_4)_2SO_4$ and 3% glycerol. X-ray diffraction data were collected on BL-5A, Photon Factory, Tsukuba, Japan. The data set was processed with *HKL*-2000 and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). The structure of *H. fusiformis* cytochrome c_6 was determined by molecular replacement using the program *MOLREP* (Collaborative Computational Project, Number 4, 1994). The search model used was

Table 1
Crystal parameters and data-collection and structure refinement.

Values in parentheses are for the outer shell (1.66–1.60 Å).

Data-collection statistics	
Temperature (K)	100
Resolution range (Å)	50.0–1.6
Space group	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 84.578$, $c = 232.911$
Reflections (measured/unique)	699906/107513 (9592)
Completeness (%)	95.6 (86.8)
R_{merge}† (%)	4.8 (23.5)
Redundancy	6.6 (3.5)
Mean $I/\sigma(I)$	20.5
Mosaicity	0.33
Refinement statistics	
Resolution range (Å)	20.0–1.6
σ Cutoff/reflections used	0.0/107239
R factor/R_{free}‡ (%)	18.4/20.9
R.m.s.d. bond lengths (Å)/bond angles (°)	0.011/1.165
B factors (Å^2)	
Average	22.4
Protein	21.1
Haem	14.8
Water	34.0
Sulfate	40.5
Ramachandran plot	
Residues in most favourable region (%)	82.7
Residues in additional allowed region (%)	16.0
Residues in disallowed region (%)	1.3

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an observation and $\langle I(hkl)\rangle$ is the mean value for the unique reflection; summations are over all reflections. ‡ R factor $= \sum_h |F_o(h) - F_c(h)| / \sum_h F_o(h)$, where F_o and F_c are the observed and calculated structure-factor amplitudes, respectively. The free R factor was calculated using 5% of the data, which were excluded from the refinement.

Porphyra yezoensis cytochrome c_6 (Yamada *et al.*, 2000). The structure of *H. fusiformis* cytochrome c_6 was refined with *REFMAC* from the *CCP*4 program suite. Water molecules were added using a water-pick script in *CNS* and refinement was continued using *REFMAC*5 (Collaborative Computational Project, Number 4, 1994). The final model obtained had an R factor of 18.4% and a free R factor of 20.9%. Manual model building was carried out using *Coot* (Emsley & Cowtan, 2004). Solvent molecules were placed at positions where spherical electron-density peaks were found above 1.5σ in the $|2F_o - F_c|$ map and above 3.0σ in the $|F_o - F_c|$ map and where stereochemically reasonable hydrogen bonds were allowed. A summary of the data-collection and refinement statistics is given in Table 1.

3. Results and discussion

3.1. Sequence of *H. fusiformis* cytochrome c_6

To elucidate the genome code of a cytochrome c_6 gene from a brown alga, we determined the protein cDNA from the brown alga *H. fusiformis* as shown in Fig. 1 (Genbank accession No. AB105058). *H. fusiformis* cytochrome c_6 genes were amplified using cDNA, which was performed by the reverse transcription of poly(A)$^-$ mRNA. The polyadenylation signal sequences (AAUAAA) necessary for the addition of polyadenylic acid were not included in the 3′-region of the cytochrome c_6 gene from *H. fusiformis*, but the 3′-regions of the cDNA of the cytochrome c_6 that contained the sequence that can form a stem-loop structure that stabilizes mRNA were transcribed from the chloroplast genome (Drager *et al.*, 1996; Yang & David, 1997). The gene that was transcribed from the chloroplast genome does not add polyadenylic acids (Sagher *et al.*, 1976). Generally, the addition of polyadenylic acids that participate in mRNA stability occurs after transcription inside the nucleus (Darnell *et al.*, 1971; O'Hara *et al.*, 1995). The Shine–Dalgano (SD) sequence, a 16S-ribosomal RNA-binding site that is rich in purine 3–9 bases upstream of the initiation codon of prokaryotic cell mRNA (Bonham-Smith &

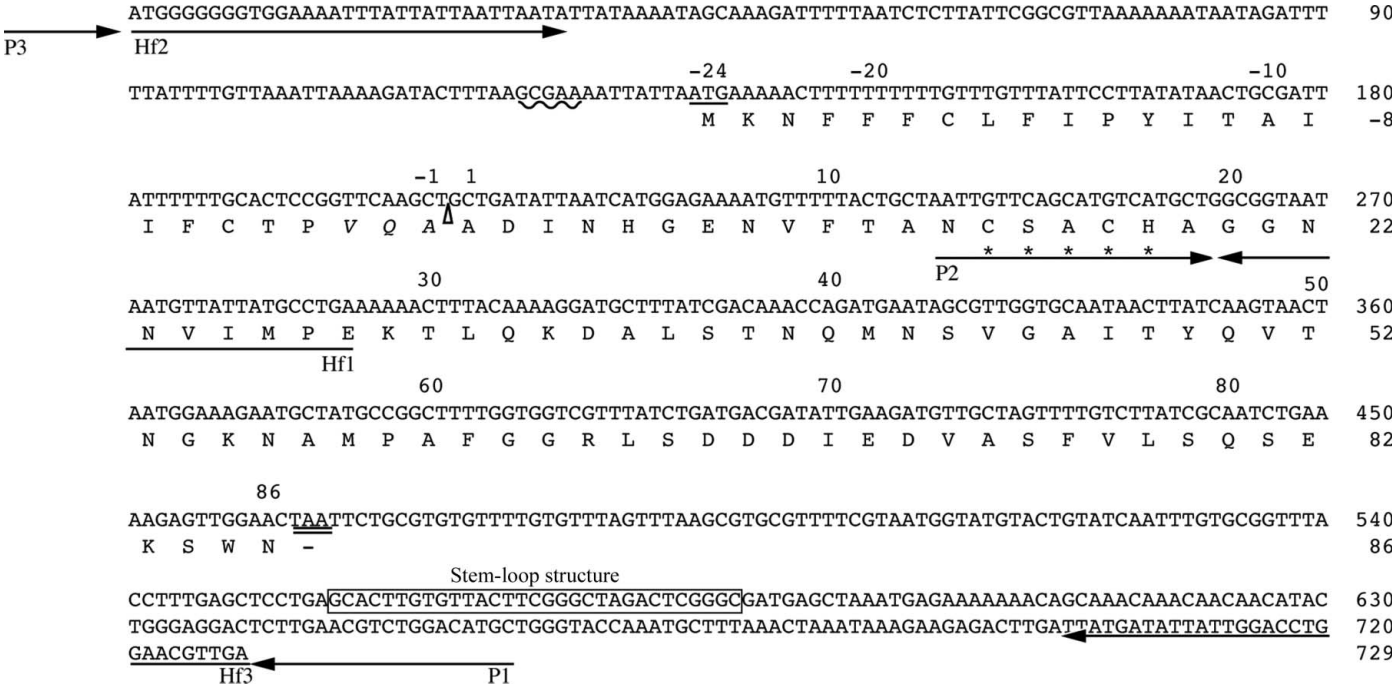

Figure 1
Nucleotide sequence and deduced amino-acid sequence of the cDNA encoding cytochrome c_6 from the brown alga *H. fusiformis*. The amino-acid residues numbered −1 to −24 and 1–86 constituted the putative transit sequence and the mature peptide sequence, respectively. Underlined and double-underlined nucleotides indicate the initiation codon and termination codon, respectively. Nucleotides underlined with a wavy line indicate the Shine–Dalgarno-like sequence. The open arrowhead indicates the putative transit-peptide cleavage site. The arrows indicates the primers used. The residues marked with asterisks and the amino-acid residues in italics (−1 to −3) correspond to the haem *c* motif (Cys-*X*-*X*-Cys-His) and cleavage motif (Val-*X*-Ala), respectively. The box indicates the stem-loop structure-formation sequence.

Bourque, 1989), was present ten bases upstream of the initiation codon in the *H. fusiformis* cytochrome c_6 gene (Fig. 1). There were also no SD sequences in the cytochrome c_6 genes of the green alga *C. reinhardtii* (Merchant & Bogorad, 1987), the euglena *Euglena gracilis* (Vacula *et al.*, 1999) and the cyanelle *Cyanophora paradoxa* (Steiner *et al.*, 2000) that are encoded in the nuclear genome. We obtained a genomic DNA clone of approximately 730 bp that was amplified using primers constructed based on cDNA sequences (Fig. 1). The gene that encodes cytochrome c_6 was not inserted with an intron. The green alga *C. reinhardtii* gene encoding cytochrome c_6 has been reported to have its coding region interrupted by two introns (Hill *et al.*, 1991). Genes encoded in nuclear genomes are usually inserted with introns (The Arabidopsis Initiative, 2000), but genes encoded in chloroplast genomes do not have these insertions (Shinozaki *et al.*, 1986). These results showed that the cytochrome c_6 gene from the brown alga *H. fusiformis* was encoded in the chloroplast genome. At present, only red and brown algae have been reported to have a cytochrome c_6 gene encoded in the chloroplast genome.

3.2. Protein expression and purification

E. coli BL21 (DE3) harbouring both pET22bHfc6 and pSTV28*ccmA–H* was used as a source of recombinant *H. fusiformis* cytochrome c_6. Recombinant *H. fusiformis* cytochrome c_6 was purified by ammonium sulfate pecipitation and two-step anion-exchange chromatography. The degree of homogeneity was confirmed by tricine SDS–PAGE and UV–visible spectroscopy. After initial purification by anion-exchange chromatography, tricine SDS–PAGE analysis displayed a predominant cytochrome c_6 band and a minor band and the fractions with an $A_{275}/A_{552.5}$ ratio lower than 2.0 were pooled and concentrated. After a second chromatography purification step, tricine SDS–PAGE analysis showed only the cytochrome c_6 band and the purification ratio ($A_{275}/A_{552.5}$) of *H. fusiformis* cytochrome c_6 was 0.90, which was similar to that of other cytochromes c_6.

The protein consists of 86 amino acids and one *c*-type haem and its molecular weight was calculated to be 9762.4 Da. From SDS–PAGE analysis a value of 8.0 kDa was obtained, which is somewhat lower than that deduced from the sequence (Fig. 2*a*). Similar discrepancies have been observed in other small negatively charged proteins, such as the green alga *Monoraphidium braunii* cytochrome c_6 and plastocyanin (Campos *et al.*, 1993).

The UV–visible spectra of reduced and oxidized recombinant *H. fusiformis* cytochrome c_6 are shown in Fig. 2(*b*). In the reduced form, the α, β, γ (Soret) and δ absorption maxima peaks appear at 552.5, 522.0, 416.0 and 318.0 nm, respectively. For the oxidized form of the cytochrome c_6, the $\alpha + \beta$ and γ (Soret) absorption maxima peaks were 528.0 and 408.5 nm, respectively; a shoulder peak at 695.0 nm, indicating His–Fe–Met coordination, was observed (Fig. 2*b*, inset).

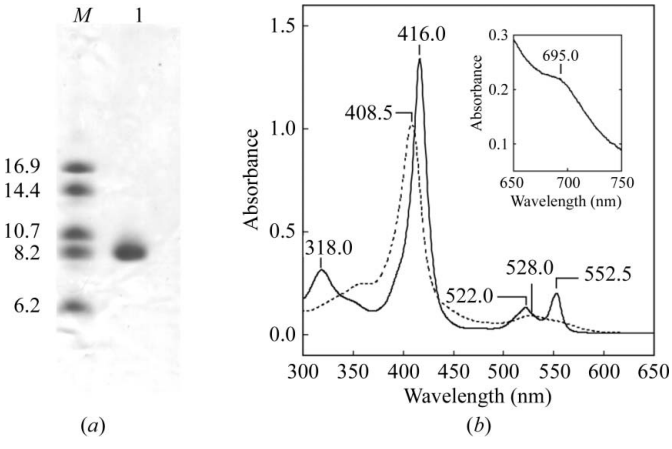

Figure 2
SDS–PAGE analysis and UV–visible spectra of purified *H. fusiformis* cytochrome c_6. (*a*) Proteins were analysed on 16.5% tricine SDS–PAGE and stained with Coomassie Blue. Lane *M*, molecular-weight markers (kDa); lane 1, purified *H. fusiformis* cytochrome c_6. (*b*) UV–visible spectra of dithionite-reduced (solid line) and ferricyanide-oxidized (dotted line) forms of *H. fusiformis* cytochrome c_6 (10 μM) were measured in 10 m*M* sodium phosphate pH 7.0 at 298 K. The inset shows the 695 nm band of the oxidant form at 500 μM.

3.3. Crystallization of *H. fusiformis* cytochrome c_6

A crystallization droplet was prepared by mixing 2 µl protein solution (20 mg ml^{-1} protein) in 10 m*M* sodium phosphate buffer pH 7.0 and 2 µl reservoir solution consisting of 0.1 *M* CAPS pH 10.5, 0.2 *M* Li_2SO_4, 2.2 *M* $(NH_4)_2SO_4$ and 3% glycerol and was equilibrated against 500 µl of the same reservoir solution at 293 K. Diffraction-quality crystals appeared within a week (Fig. 3). This reservoir solution used for *H. fusiformis* cytochrome c_6 is somewhat similar to that used for cytochrome c_6 from the cyanobacterium *Arthrospira maxima* [reservoir solution containing 0.1 *M* Tris pH 7.8, 0.2 *M* Li_2SO_4, 2.2 *M* $(NH_4)_2SO_4$ and 1% glycerol (Sawaya *et al.*, 2001)], but few similarities were found between the reservoir solutions used for *H. fusiformis* cytochrome c_6 and those used for other algal and cyanobacterial cytochromes c_6 (Kerfeld *et al.*, 1995; Frazão *et al.*, 1995; Schnackenberg *et al.*, 1999; Yamada *et al.*, 2000; Dikiy *et al.*, 2002; Worrall *et al.*, 2007).

3.4. Overall structure of *H. fusiformis* cytochrome c_6

The crystal structure of *H. fusiformis* cytochrome c_6 has been determined at 1.6 Å resolution. This is the first cytochrome c_6 crystal structure for a brown secondary symbiotic alga. The crystal belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 84.58$, $c = 232.9$ Å and six molecules (*A*, *B*, *C*, *D*, *E* and *F*) per asymmetric unit (Fig. 4*a*). These six molecules could be superimposed with main-chain root-mean-square deviation (r.m.s.d.) values of 0.1–0.4 Å, as determined using the *DALI* program (Holm & Park, 2000). The hexamer contains four sulfate ions which may be derived from the ammonium sulfate and lithium sulfate included in the crystallization solution. The cytochrome c_6 hexamer was formed of a dimer of trimers (*ABC* and *DEF* trimers; Fig. 4*b*). An intermolecular hydrogen bond was formed in the *ABC* trimer between each pair of molecules

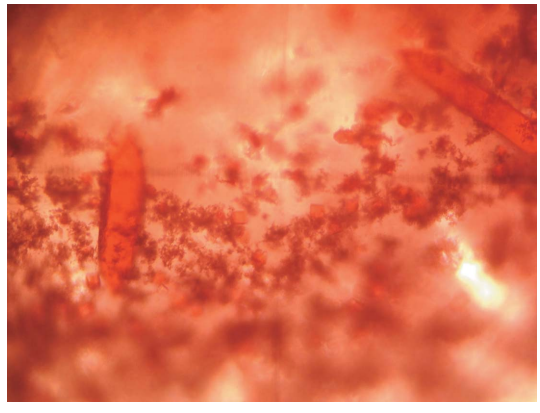

Figure 3
Crystal of *H. fusiformis* cytochrome c_6 grown in 0.1 *M* CAPS pH 10.5, 0.2 *M* Li_2SO_4, 2.2 *M* $(NH_4)_2SO_4$ and 3% glycerol.

in the trimer through Ala60 N and Arg64 O and one sulfate ion was centred between the Arg64 side chains of the three molecules (Fig. 4*c*). This arrangement of a sulfate ion enclosed by a basic amino-acid residue has been also found in the crystal structure of *Hydrogenobacter thermophilus* cytochrome c_{552} (Travaglini-Allocatelli *et al.*, 2005). Considering that the crystals of *H. fusiformis* cytochrome c_6 were obtained in the presence of sulfate ions, the sulfate ions were convenient for crystallization and might contribute to crystal-packing stabilization by neutralization of charge repulsion in this region. Therefore, we deduce that the crystallographic hexamer is a nonphysiological crystal-packing artifact. An intermolecular hydrogen bond was formed between each pair of molecules of the second trimer through the C-terminal Asn86 $N^{\delta2}$ and Asn86 OX (Fig. 4*c*). Hydrogen bonds between the two trimers were formed between Asn22 $O^{\delta1}$ of the *ABC* trimer and Arg64 $N^{\varepsilon2}$ of the *DEF* trimer.

An oligomeric arrangement of molecules has been found in the crystal structures of other cytochromes c_6. A trimeric arrangement of molecules has been found in the structures of cytochrome c_6 from *C. reinharditii* form I (Kerfeld *et al.*, 1995) and *M. braunii* (Frazão *et al.*, 1995). The proteins from *Scenedesmus obliquus* (Schnackenberg *et al.*, 1999), *A. maxima* (Sawaya *et al.*, 2001) and *Phormidium laminosum* (Worrall *et al.*, 2007) have been crystallized as dimers. These oligomers of cytochromes c_6 were formed by the packing of different molecules and were not superimposed. It has been reported that the observed differences in oligomerization between various cytochromes c_6 may be determined by subtle differences in their surface electrostatic potential properties (Dikiy *et al.*, 2002). In

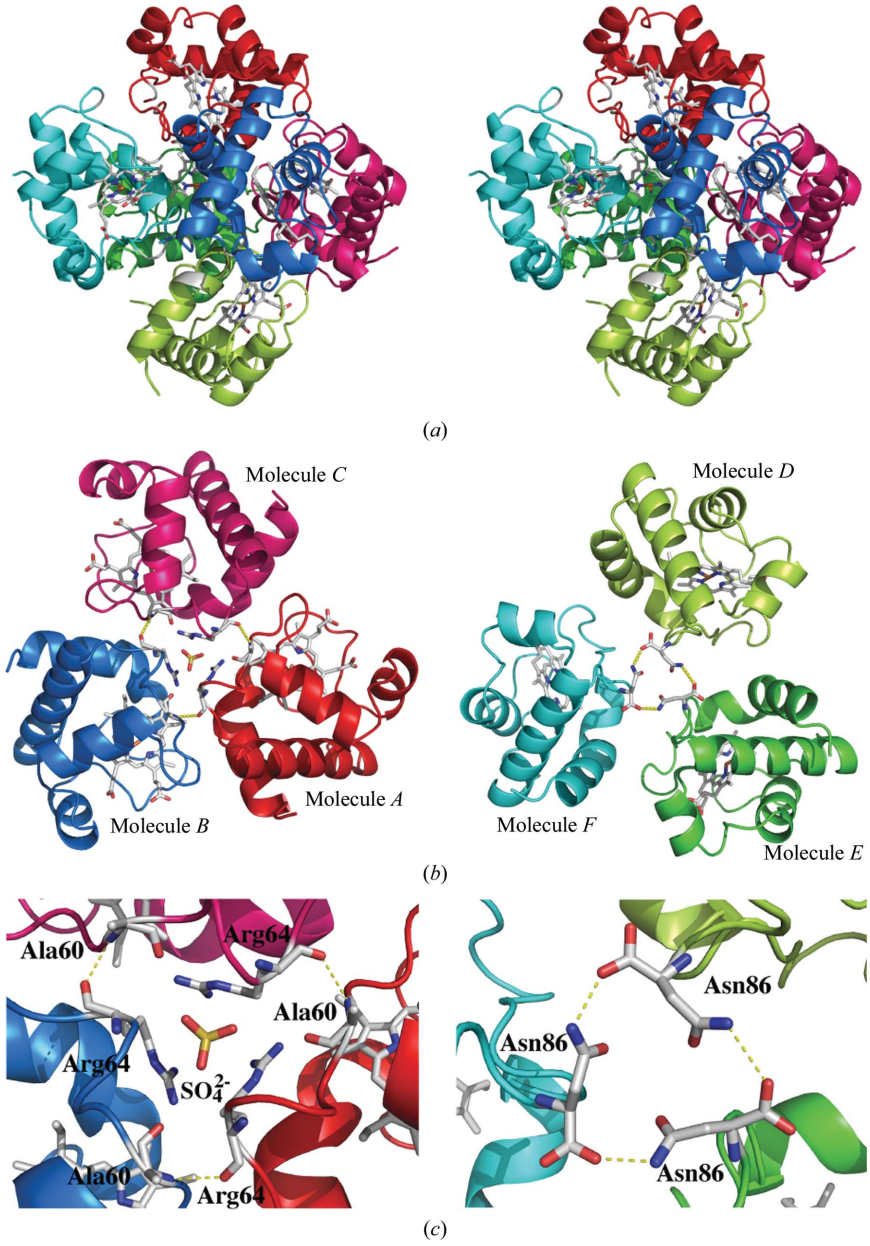

Figure 4
H. fusiformis cytochrome c_6 hexamer. Six protein molecules are displayed, with each molecule in a different colour (red, molecule *A*; marine, molecule *B*; magenta, molecule *C*; lemon, molecule *D*; green, molecule *E*; cyan, molecule *F*). The amino-acid residues and haem group are represented by a stick model with atom-specific colours: white, carbon; blue, nitrogen; red, oxygen; yellow, sulfur; iron, orange. This figure was drawn with *PyMOL*.

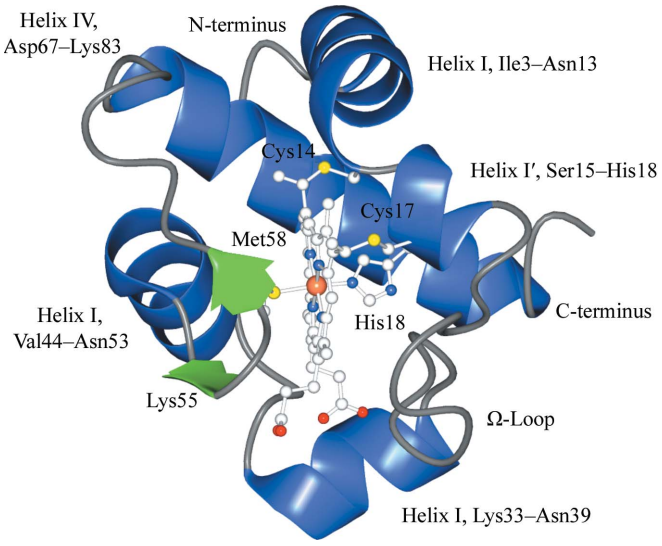

Figure 5
Overall structure of cytochrome c_6 from the brown alga *H. fusiformis*. The α-helix (marine) and β-sheet (green) are indicated as a cartoon model. Cys14, Cys17, His18, Met58 and haem are represented using a ball-and-stick model in the same colour scheme used in Fig. 1. This figure was drawn with *CCP*4 (Collaborative Computational Project, Number 4, 1994).

contrast, the cytochromes c_6 from *P. yezoensis* (Yamada *et al.*, 2000) and *Cladophora glomerate* (Dikiy *et al.*, 2002) are monomeric in the crystal.

The structure of *H. fusiformis* cytochrome c_6 belongs to the class I *c*-type cytochromes, which are composed of four α-helices and tight turns (Fig. 5). The protein consists of a single polypeptide chain folded around the haem prosthetic group. The secondary structures have been classified according to the criteria of Kabsch & Sander (1983). Four α-helices, Asp2–Asn13 (I), Ser15–His18 (I′), Lys33–Ala38 (II), Ile44–Asn53 (III) and Asp67–Lys83 (IV), are found as elements of a regular secondary structure, with helices I and IV overlapping at about 90° (Fig. 3). A two-stranded antiparallel β-sheet was formed with two interchain hydrogen bonds between Lys55 and Met58, which form a type II′ β-turn with Asn56 and Ala57. A short β-sheet has commonly been observed in the structures of cyanobacterial, green and red algal cytochromes c_6.

3.5. Structural comparison between *H. fusiformis* and other cytochromes c_6

The crystal structures of four cytochromes c_6 from the eukaryotic green algae *C. reinharditii* (Kerfeld *et al.*, 1995), *M. braunii* (Frazão *et al.*, 1995), *S. obliquus* (Schnackenberg *et al.*, 1999) and *C. glomerata* (Dikiy *et al.*, 2002), of one from the eukaryotic red alga *P. yezoensis* (Yamada *et al.*, 2000) and of two from the prokaryotic cyanobacteria *A. maxima* (Sawaya *et al.*, 2001) and *P. laminosum* (Worrall *et al.*, 2007) have been determined. They are composed of 85–90 amino acids and their main secondary-structural elements are α-helices wrapping around the haem prosthetic group. An amino-acid sequence comparison of *H. fusiformis* cytochrome c_6 with those from *C. reinharditii*, *M. braunii*, *C. glomerata*, *S. obliquus*, *P. yezoensis*, *A. maxima* and *P. laminosum* revealed similarities of 46.67, 47.78, 45.05, 47.19, 72.09, 53.33 and 59.77%, respectively (Fig. 6) and the amino-acid sequence of *H. fusiformis* cytochrome c_6 is most similar to that of *P. yezoensis* cytochrome c_6. The main-chain r.m.s.d.s between *H. fusiformis*, *C. reinharditii*, *P. yezoensis* and *A. maxima* cytochromes c_6 are 0.5–1.1 Å, as determined using the *DALI* program (Holm & Park, 2000). A C^{α} trace of *H. fusiformis* cytochrome c_6 shows a high overall similarity between the green algal and cyanobacterial cytochromes c_6, as well as subtle differences (Fig. 7). The largest deviation in the C^{α} trace between the brown alga *H. fusiformis* cytochrome c_6 and green algal and cyanobacterial cytochromes c_6 was found in the second interconnecting loop (Gln40–Ser43; Fig. 7). The green algal and cyanobacterial cytochromes c_6 have a small insertion of 2–4 amino acids in this region compared with *H. fusiformis* cytochrome c_6. The loop region in *H. fusiformis* cytochrome c_6 resembles that in cytochrome c_6 from the red alga *P. yezoensis*, which also lacks two amino acids in this region compared with green algal and cyanobacterial cytochromes c_6. Considering that the loop region of cytochromes c_6 has a poorly conserved amino-acid sequence compared with other regions, this region may have no common biological functional role. In the structure of other cytochromes, functional roles have not been reported for this region.

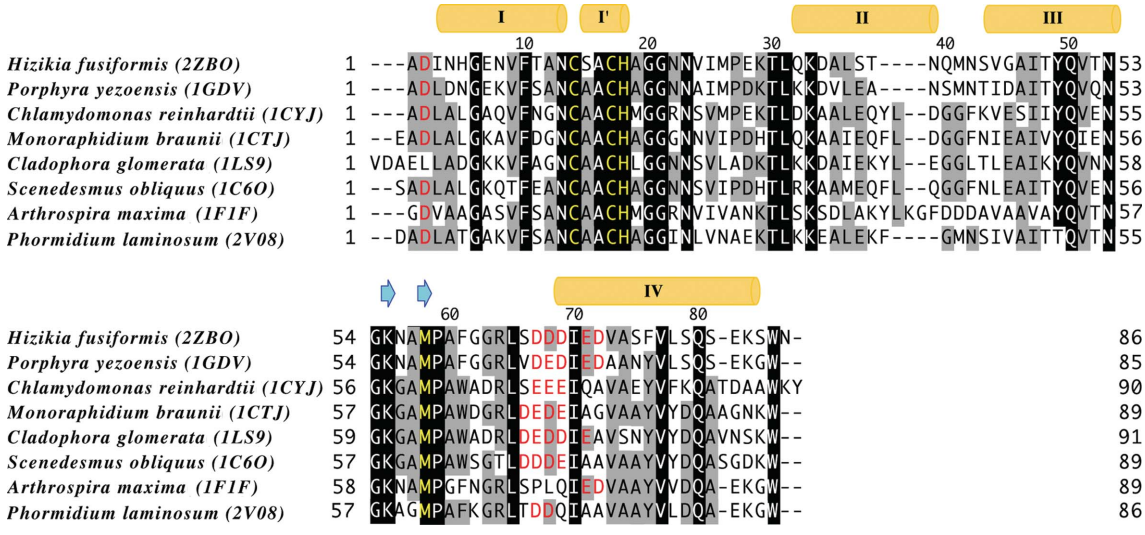

Figure 6
Aligned amino-acid sequences of cytochromes c_6 with reported crystal structures. The conserved and semi-conserved amino-acid residues among the six algal species and two cyanobacterial species are indicated by black and grey boxes, respectively. The haem ligands and the residues forming the acidic patch of the exposed surface are shown in yellow and red, respectively. The secondary structure of cytochrome c_6 from *H. fusiformis* is indicated: orange cylinders, α-helices; blue arrows, β-sheets.

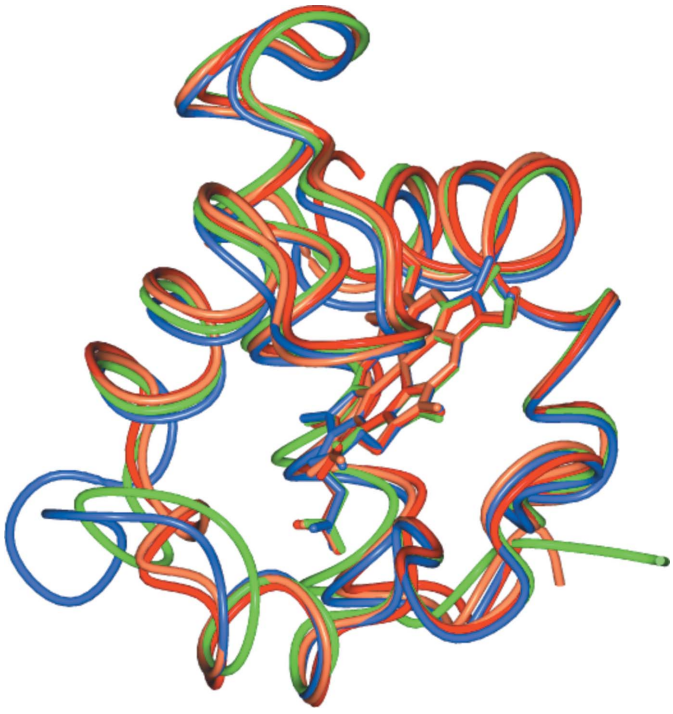

Figure 7
Superimposition of the C^{α} traces of oxidized cytochromes c_6 from the brown alga *H. fusiformis* (orange; PDB code 2zbo), the red alga *P. yezoensis* (red; PDB code 1gdv), the green alga *C. reinharditii* (green; PDB code 1cyj) and the cyanobacterium *A. maxima* (marine; PDB code 1f1f). The alignment was prepared using the *DALI* program (Holm & Park, 2000).

In this study, we showed that the cytochrome c_6 gene from the brown alga *H. fusiformis* was encoded in the chloroplast genome. To date, the cytochrome c_6 gene has only been found to be encoded in the chloroplast genome in red and brown algae. The amino-acid sequence and tertiary structure of *H. fusiformis* cytochrome c_6 were very similar to those of a red algal cytochrome c_6 rather than those of green algal cytochromes c_6. The present results support the hypothesis that brown algae gained their chloroplasts *via* secondary endosymbiosis involving a primitive red algal endosymbiont and a nonphotosynthetic eukaryote host.

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