Plant Molecular Biology **42:** 439–450, 2000.

Chlorophyll breakdown in *Chlorella protothecoides*: characterization of degreening and cloning of degreening-related genes

Stefan Hörtensteiner[1,*], Joanne Chinner[2], Philippe Matile[1], Howard Thomas[2] and Iain S. Donnison[2]

[1]*Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland (*author for correspondence); [2]Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Ceredigion, SY23 3EB, UK*

Received 17 May 1999; accepted in revised form 9 November 1999

Key words: cDNA, *Chlorella protothecoides*, chlorophyll breakdown, degreening, differential screening, senescence

Abstract

Chlorella protothecoides cultures grown in a nitrogen-free bleaching medium (BM−N) in the dark rapidly degraded chlorophyll (Chl) to red catabolites. This degreening process was investigated under different growth conditions. Supply of nitrogen to the culture medium (BM+N) inhibited bleaching and the synthesis of catabolites as did the addition to BM−N of cycloheximide or a chelator, 2,2′-bipyridyl. In contrast, chloramphenicol or the protease inhibitor E64 had no effect. During bleaching, Chl breakdown was accompanied by the degradation of cellular proteins such as light-harvesting complex II, cytochrome *f* and protochlorophyllide oxido-reductase. During growth in BM–N, protease activity increased and proteins immunologically detectable with an antibody against a senescence-enhanced cysteine protease accumulated. cDNAs from BM−N and BM+N cells were used for differential and subtractive screening to isolate cDNAs representing genes with degreening-enhanced expression (*dee*) in *C. protothecoides*. Several different *dee*s were identified with different patterns of expression during *Chlorella* growth but which were all expressed at higher levels during bleaching. Among these, *dee*4 was most abundant and its expression was exclusive in BM–N cultures. Analysis of the *dee* sequences showed that they encode different proteins including a novel amino acid carrier (*dee*4), ferritin, ATP-dependent citrate lyase, a Ca^{2+}-binding protein, MO25, ubiquinone-cytochrome *c*-reductase and several new proteins.

Introduction

Chlorophyll breakdown is the most conspicuous symptom of leaf senescence and fruit ripening and has, therefore, been widely used as a marker to monitor senescence. It is not only important for the understanding of a fundamental biological process, but also has substantial economic impact (Brown *et al*., 1991). For example, in the food industry, inhibition of Chl breakdown during the processing of 'green vegetables' is desired (Heaton and Marangoni, 1996). On the other hand, degreening is an essential part of normal ripening in many fruits and interference with the process can lead to practical problems. For instance, in banana peel ripening, which is accompanied by desirable degreening, elevated temperatures inhibit Chl breakdown so that harvested fruits require shipping and storage at below 24 °C (Seymour *et al*., 1987). Chl catabolism can also have value for tourism. For example, the autumnal colour change of deciduous trees during the 'Indian summer' brings many visitors to the northeast of the USA every year (Hendry *et al*., 1987). Conversely, the inhibition of breakdown is important in the maintenance of green lawns in the

The nucleotide sequence data reported will appear in the EMBL database under the accession numbers AJ238625 (*dee*8), AJ238626 (*dee*10), AJ238627 (*dee*112), AJ238628 (*dee*188), AJ238629 (*dee*189), AJ238630 (*dee*138), AJ238631 (*dee*22), AJ238632 (*dee*76), AJ238633 (*dee*165), AJ238634 (*dee*25) and AJ238635 (*dee*4).

leisure industry such as for golf or soccer pitches. Yet, despite the biological and economic importance of Chl degradation, the underlying biochemical processes have only recently been described (for a review, see Matile *et al*., 1999) and only one gene for an enzyme of the catabolic pathway, chlorophyllase, has been cloned (E.E. Goldschmidt, unpublished). In contrast, the pathway of Chl biosynthesis has been fully elucidated and genes have been identified for all of the enzymes involved (for a review, see von Wettstein *et al*., 1995).

Chl catabolism during senescence proceeds in several steps starting with the removal of phytol and central Mg atom by chlorophyllase and Mg dechelatase, respectively (Matile *et al*., 1996). Subsequently, the chlorin macrocycle of Pheide *a* is cleaved by Pheide *a* oxygenase and red Chl catabolite (RCC) reductase to form a primary fluorescent catabolite, pFCC. This key step in Chl breakdown is remarkable as it is catalysed by two proteins acting in a channelled reaction with RCC as the intermediate (Rodoni *et al*., 1997). Pheide *a* oxygenase is located in the inner envelope membrane of gerontoplasts (senescent chloroplasts) (Matile and Schellenberg, 1996) and specifically incorporates an oxygen atom, derived from dioxygen, in the formyl group attached to ring B of pFCC (Hörtensteiner *et al*., 1998) (Figure 1). Elucidation of the structures of nonfluorescent Chl catabolites (NCCs), the final products of Chl degradation, from various higher plant species (Kräutler *et al*., 1991; Mühlecker *et al*., 1993; Iturraspe *et al*., 1995; Curty and Engel, 1996; Mühlecker and Kräutler, 1996) has revealed two additional common reactions to occur during pFCC to NCC transformation, i.e. hydroxylation at C8 and tautomerization in the ring D and the γ methine bridge. Further species-specific modifications of NCCs are also possible. Of the Chl catabolic enzymes investigated, only the activities of Pheide *a* oxygenase (Schellenberg *et al*., 1993; Hörtensteiner *et al*., 1995) and Chl *b* reductase (converting Chl *b* to *a*) (Scheumann *et al*., 1999) have been demonstrated to be senescence-specific.

In *Chlorella protothecoides*, degreening can be induced by growing cells heterotrophically in the dark without a source of nitrogen. Under these conditions, the algae excrete red bile pigments into the surrounding medium (Oshio and Hase, 1969). Structure elucidation of the major pigments (Engel *et al*., 1991, 1996) confirmed their origin from Chl and, in contrast to higher plants, where all NCCs so far identified are exclusively derived from Chl *a*, one of

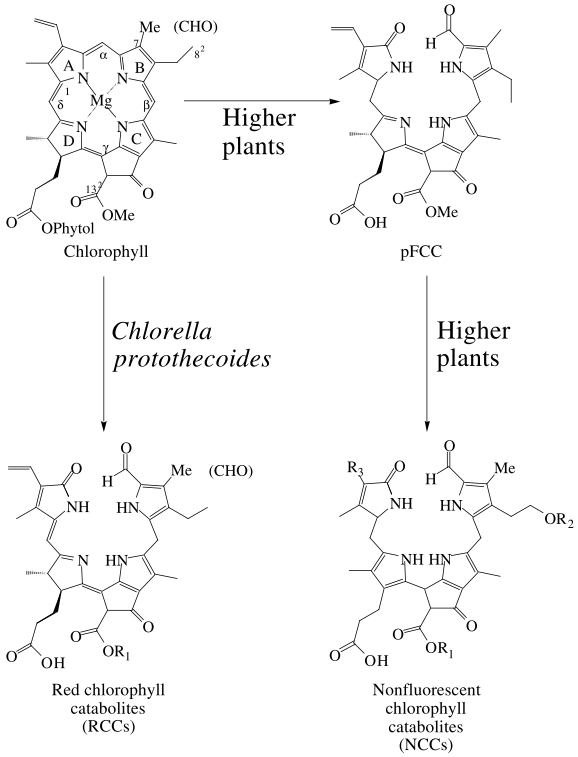

Figure 1. Chl and Chl catabolites occurring during degreening in *Chlorella protothecoides* and higher-plant senescence, respectively. R_1 = H or CH_3. R_2, R_3 indicate differences in the chemical constitutions of NCCs identified from higher plants (for a review, see Matile *et al*., 1999). Pyrrole rings (A–D), methine bridges (α-δ) and relevant carbon atoms are labelled. Substitution of formyl (CHO) for methyl (Me) at C(7) forms Chl *b* from Chl *a*.

the *Chlorella* catabolites has been found to have a *b* configuration (Iturraspe *et al*., 1994). The major red degradation product (Figure 1) is structurally identical to the RCC of higher plants. Moreover, the mechanism of chlorin ring opening is identical in both higher-plant and algal systems with respect to the incorporation of molecular oxygen (Curty *et al*., 1995; Hörtensteiner *et al*., 1998). This suggests that the oxygenase which catalyses bile pigment formation in *Chlorella* is functionally equivalent to the Pheide *a* oxygenase of higher plants. Although neither the substrate nor the electron donor(s) of the ring cleavage reaction in *Chlorella* have been identified, it can be speculated that the principal features are the same as in higher plants.

In senescing leaves, Chl catabolism is accompanied by the degradation of proteins, nucleic acids and lipids, and the export of nutrients, particularly nitrogen (for a review, see Matile, 1992). The ultrastructural organization of senescing cells is significantly

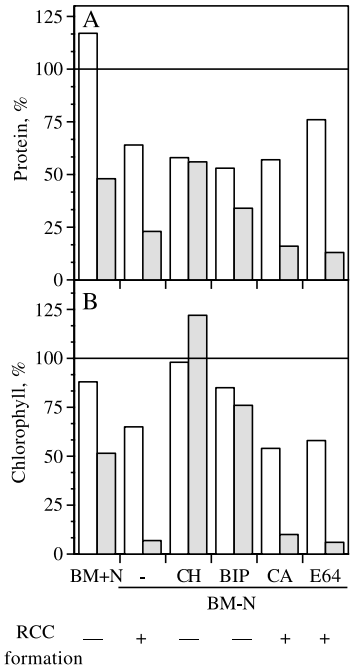

Figure 2. Relative protein (A) and Chl (B) contents of *Chlorella protothecoides* cells during incubation for 24 h (white columns) and 48 h (dashed columns) in BM+N, BM−N, and BM−N supplemented with cycloheximide (CH, 0.1 mM), 2,2′-bipyridyl (BIP, 1 mM), chloramphenicol (CA, 0.1 mM) and E64 (0.1 mM), respectively. Contents before incubation were set at 100% corresponding to 1.22 μg protein and 89.5 ng Chl 10^{-7} cells, respectively.

altered by the dismantling of chloroplasts and other organelles and finally leads to vacuolar membrane rupture and autolysis (Fukuda, 1996; Matile, 1997). In contrast to senescence of higher plants, processes accompanying the bleaching of *Chlorella* cultures have received little attention (Shihira-Ishikawa and Hase, 1964). A promising approach to investigate the molecular changes involved in degreening of *Chlorella* is to use a differential or subtractive screening method. Such techniques have been used to identify senescence-related genes in a number of higher plants (for a review, see Buchanan-Wollaston, 1997). From these studies, genes have been isolated which code for proteins that are involved in protein degradation (e.g. Smart *et al*., 1995; Buchanan-Wollaston and Ainsworth, 1997) and in protection against oxidative damage and other stresses (e.g. Hanfrey *et al*., 1996; Buchanan-Wollaston, 1997).

In this report we show that *Chlorella protothecoides* represents a promising model system for the study of Chl catabolism. By differential and subtractive screening we have isolated cDNAs with enhanced expression during degreening in *Chlorella protothecoides*.

Materials and methods

Plant material

Chlorella protothecoides strain ACC25 (Shihira-Ishikawa *et al*., 1964; Engel *et al*., 1991) was grown in a 'greening' medium (GM) comprising 10 mM K_2HPO_4, 10 mM KH_2PO_4, 15 mM $(NH_4)_2HPO_4$, 1.2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM $FeSO_4$ and 1 ml/l Arnon A5 microelement solution (Arnon, 1938). After autoclaving, 2 ml/l Nitsch and Nitsch vitamins (Sigma) and 0.5% w/v glucose were added. Algae were maintained by weekly inoculation (1:50) of fresh medium and grown under permanent illumination using fluorescent light (2000–3000 lx) at 25 °C. For experiments, fresh cultures (each of 50 ml) were grown for 3–4 days in the light. Cells were then collected by centrifugation (1600 × *g*, 4 min) and washed twice in 20 ml of 0.1 M potassium phosphate (K-Pi) buffer pH 7.0. After washing, cells were resuspended in 1 ml of K-Pi buffer, and transferred to 50 ml bleaching medium (BM). The BM was the same as GM, except that it contained 0.1 M K_2HPO_4, 0.1 M KH_2PO_4 and 5% w/v glucose. BM either contained 15 mM $(NH_4)_2HPO_4$ as above or was devoid of a nitrogen source (BM+N or BM−N, respectively). In some experiments, chemicals were added to BM–N as indicated in the figures. After growth in permanent darkness at 25 °C for a range of times between 0 and 66 h, cells were collected (1600 × *g*, 4 min), resuspended in 10 mM potassium-phosphate pH 7.0 and frozen in liquid nitrogen before storage at −80 °C.

Extraction and analysis of pigments

Frozen *C. protothecoides* cells were thawed in 80% acetone and immediately lysed by shaking at maximum speed for 4 × 30 s in a FastPrep Instrument (BIO 101, La Jolla, CA) using FastDNA tubes. The pigments were separated from precipitated proteins by centrifugation (14 000 × *g*, 5 min, 4 °C) and the supernatant directly employed for spectrophotometric determination of Chl (Lichtenthaler, 1987) and for analysis of green pigment composition with reversed-phase HPLC (Langmeier *et al*., 1993).

Extraction and analysis of proteins

C. protothecoides cells (400 μl) were treated with 2 volumes of LE buffer (50 mM H_3PO_4-LiOH pH 8.0, 1 mM lithium iodoacetate, 5% w/v glycerol, 1% v/v 2-mercaptoethanol, 0.17 g/l phenylmethanesulfonylfluoride) and lysed in a FastPrep Instrument as described above. Between the shaking steps, the tubes were cooled on ice for 1 min. After centrifugation for 5 min at 14 000 $\times$ *g* (4 °C), to remove insoluble material, the protein content was determined according to Bradford (1976) with BSA as a standard. To the remainder of the protein extract, lithium dodecyl sulfate was added to a final concentration of 1.6%, samples were heated to 95 °C for 1 min and proteins separated by sodium dodecyl sulfate (SDS) polyacrylamide (12.5%) gel electrophoresis (Bachmann *et al*., 1994). After western blotting, detection was either by immuno-labelling (Hilditch *et al*., 1989) with peroxidase-conjugated secondary antibodies or the ECL Plus Western blotting detection system (Amersham). Primary antibodies were raised in sheep (Cyt *f*) or rabbit (LHCII, POR and see1) against proteins of *Arabidopsis* (POR; Sperling *et al*., 1997) and *Festuca* (LHCII, Hilditch, 1986; and Cyt *f*, Davies *et al*., 1989), or against a synthetic peptide of maize see1 (I.S. Donnison, C.M. Griffiths and H. Thomas, unpublished).

Protease assay

Protease activity was measured in a plate-based assay with 0.24% w/v gelatin as substrate. In addition to gelatin, the plate contained 0.3 M K-Pi buffer at pH 5.8, 6.8 and 8.0, respectively, reduced glutathione (1.2 mg/ml) and 1.2% w/v agarose. All the ingredients were heated to melt the agarose and gelatin, and 5 ml poured into a 90 mm petri plate. After the addition to the plates of *Chlorella* cells which had been lysed in K-Pi buffer using a FastPrep Instrument as described above, the plates were sealed and incubated at 30 °C overnight. The protein was stained using amido black and after destaining protease activity could be visually detected by cleared zones on the plate.

Isolation of RNA and northern analysis

After grinding *C. protothecoides* cells in liquid nitrogen, total RNA was extracted according to a modified protocol (Schünmann *et al*., 1994) of the hot phenol method (de Vries *et al*., 1982). RNA content and purity were determined using a spectrophotometer. For each sample, 7 μg of total RNA was fractionated in a 1.5% agarose gel containing formaldehyde and transferred onto Hybond N membranes (Amersham) according to standard procedures (Sambrook *et al*., 1989). Isolated cDNA fragments to be used as probes were labelled with ^{32}P using a High Prime reaction mixture (Roche, Germany). Prehybridization and hybridization were performed essentially as described (Smart *et al*., 1995) except that SSC was substituted for SSPE (Sambrook *et al*., 1989).

Complementary DNA library construction and (differential) plaque screening

Using a mRNA Purification kit (Pharmacia, St. Albans, UK) poly(A)$^+$ RNA was selected from pooled total RNA samples isolated from cells grown in BM−N for 23, 39 and 66 h. Double-stranded cDNA was synthesized (Pharmacia), ligated to *Eco*RI/*Not*I adaptors, cloned into λgt11 and packaged with a Gigapack II Gold packaging extract (Stratagene, Cambridge, UK). The primary library was amplified once (Sambrook *et al*., 1989). The cDNA library was differentially screened by transferring the library onto HybondN$^+$ (Amersham) membranes and hybridizing with ^{32}P-labelled cDNA from either BM−N or BM+N cells (see below).

Subtractive cDNA analysis

Total RNA was isolated from *Chlorella* cells grown for 50 h in BM−N or BM+N with a FastRNA Green-Kit (BIO 101) according to the manufacturer's instructions and cDNA was synthesized (Pharmacia). Complementary DNAs being present in degreening (BM−N) but less abundant or absent from non-degreening (BM+N) cultures were isolated by DNA subtraction using a modified protocol (Thomas *et al*., 1997) of representational difference analysis (RDA) (Lisitsyn *et al*., 1993). BM−N and BM+N cDNAs were digested with *Dpn*II and adaptors were ligated to the fragments. After PCR amplification, BM−N and BM+N cDNAs were subjected to three rounds of RDA.

Subtraction products, following the 2nd and 3rd rounds of subtraction, were cloned into *Bam*HI-restricted pBluescript II KS (Stratagene). Insert DNA of transformants was PCR-amplified, separated by gel electrophoresis and Southern-blotted. Two replica blots were made and one screened with ^{32}P-labelled BM−N cDNA and the other screened with ^{32}P-labelled BM+N cDNA. This step

allowed us to reject false-positives generated during the RDA. Clones were further screened for degreening-enhanced/specific expression as follows. Equal amounts of BM−N and BM+N cDNAs were separated by gel electrophoresis, Southern-blotted, and probed with digoxigenin-labelled (Dig-High Prime, Roche) plasmid-derived DNA of the respective clones.

In order to identify longer cDNA clones, cDNA fragments obtained by subtractive screening were used as probes to screen the *Chlorella* degreening cDNA library. The library was transferred onto Hybond N^+ (Amersham) membranes, hybridized with ^{32}P-labelled probes (High Prime, Boehringer) and washed at 65 °C according to standard procedures (Sambrook *et al*., 1989). Positive plaques were picked, their size determined by PCR with λgt11-specific forward and reverse primers, and plaques containing the largest inserts were rescreened to isolate individual clones.

Sequencing and sequence analysis

Plasmid DNA or PCR-amplified λ phage DNA was sequenced by the dideoxynucleotide chain termination method (Sanger *et al*., 1977) with a Thermosequenase dye terminator reagent mix (Amersham) and an ABI 373A DNA sequencer (Applied Biosystems). Sequences were assembled and analysed using the Genedoc sequence analysis package (Nicholas *et al*., 1997). DNA sequences and derived protein sequences were compared to the GenBank/EMBL and the Swissprot/Trembl databases, respectively, using the BLAST program from the GCG package (Genetic Computer Group, University of Wisconcin, Madison).

Results

Changes in pigment and protein content as affected by incubation conditions

Chlorella protothecoides cells were grown in GM for 4 days and then transferred to BM+N, BM−N, or BM−N supplemented as indicated in the legend to Figure 2. After two days of culture, *Chlorella* grown in BM+N were pale green, but *Chlorella* grown in BM−N were lacking Chl. Red bile pigments (RCCs) accumulated in BM−N, but were not present in BM+N cultures. The addition of cycloheximide (CH), an inhibitor of cytoplasmic protein biosynthesis, to BM−N cultures completely inhibited degreening and the formation of RCCs. In contrast, neither chloramphenicol (CA), which inhibits plastidal protein synthesis, nor E64, a potent inhibitor of cysteine proteases, could inhibit degreening. 2,2′-Bipyridyl (BIP), which has previously been shown to create a *stay-green* phenotype during senescence in oilseed rape (Langmeier *et al*., 1993) and *Festuca* (Vicentini *et al*., 1995), completely inhibited Chl degradation and the formation of bile pigments in *Chlorella*.

Analysis of total Chl and protein contents of BM–N cultures revealed that they decreased by 77% and 93%, respectively, within 2 days of transfer to this medium (Figure 2). Similar values were obtained when E64 or CA was added to BM−N cultures, indicating that these compounds had no effect on the degreening process in *Chlorella*. In contrast, the inhibition of Chl degradation by the addition of CH or BIP was accompanied by a higher retention of total protein compared to conditions without these inhibitors. In BM+N cultures, both Chl and protein contents decreased by about 50% after 2 days of transfer. Under conditions where Chl breakdown was partially (BM+N) or completely (BM−N with CH or BIP) inhibited, HPLC analysis of pigment extracts showed that neither Chlide nor Pheide accumulated (data not shown).

Western blotting and proteolytic activity

Protein extracts from cells grown for 0, 19, 25 and 46 h in BM−N or BM+N were separated by SDS-PAGE, blotted onto nitrocellulose and probed with antibodies for LHCII, Cyt *f* or POR. All three antibodies cross-reacted with the respective *Chlorella* proteins as demonstrated in Figure 3. Whereas LHCII (A) and Cyt *f* (B) decreased during Chl breakdown in BM−N cultures, they were retained in BM+N cultures. A similar result was obtained for POR (Figure 3C), although in *Chlorella* the antibody, derived from an *Arabidopsis* sequence, cross-reacted with an additional protein apparent after growth of *Chlorella* in BM. These results indicate that during degreening in *Chlorella*, Chl-binding (LHCII) and other cellular proteins are degraded, whereas the inhibition of Chl breakdown and RCC formation in the presence of a nitrogen source in BM causes retention of individual proteins.

Figure 4A presents the result of a western blot of *Chlorella* proteins probed with an antibody raised against a peptide of maize see1 representing a sequence close to the active site domain conserved in

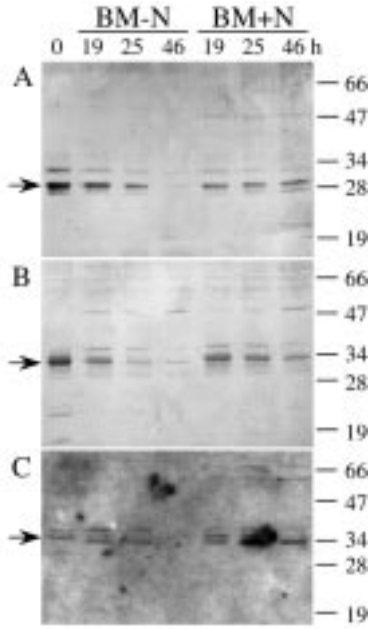

Figure 3. Western blots of total proteins of *Chlorella protothecoides* extracted after 0, 19, 25, and 46 h growth in BM−N and BM+N, visualized with antibodies against LHCII (A), Cyt *f* (B), and POR (C). The respective signals are indicated by arrows. Molecular masses (right-hand y-axis) are in kDa.

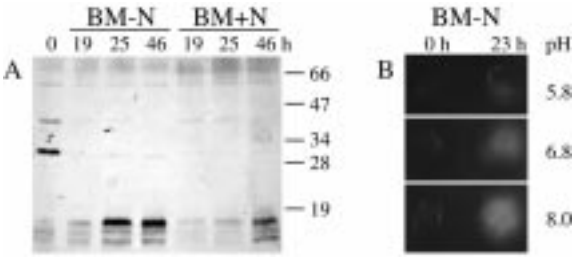

Figure 4. A. Western blot of total proteins of *Chlorella protothecoides* extracted after 0, 19, 25, and 46 h growth in BM−N and BM+N, respectively, and visualized with an antibody against see1. Molecular masses are in kDa. B. Gel protease assay of *Chlorella* proteins extracted after 0 and 23 h growth in BM−N. The pH of the respective gelatin plates is indicated.

see1 and many other similar cysteine proteases. Thus, protein bands detected in the 0 h sample may result from cysteine proteases already present in autotrophically grown *Chlorella* cells. Proteins cross-reacting with the antibodies accumulated to a higher concentration when Chl breakdown was occurring (BM−N), compared to non-degreening conditions (BM+N). The nature of the labelled proteins remains unclear, but in gel-based protease assays with *Chlorella* cell extracts (Figure 4B) protease activity was found to increase during degreening.

Construction of a cDNA library and subtractive screening

Both differential screening and RDA were used to isolate cDNAs with enhanced expression during Chl breakdown in *Chlorella protothecoides*. A cDNA library was constructed employing combined RNA samples isolated after 23, 39 and 66 h of degreening in BM−N. The library was cloned into λgt11 and contained 6×10^5 individual plaque-forming units (pfu). Complementary DNAs isolated from 50 h BM−N and BM+N cultures were used to differentially screen 2×10^5 pfu. A total of 64 putative degreening-specific plaques were identified and subsequently re-screened. After the second screen, 25 degreening-specific plaques were identified and characterized by sequencing (see below).

The RDA comprised several rounds of hybridization of BM–N cDNA with an increasing excess of BM+N cDNA followed by PCR amplification. The method selects for cDNAs that represent genes of *C. protothecoides* showing specific or enhanced expression during degreening. The average size of the RDA clones was about 250 bp and is a consequence of the initial digestion of cDNA with *Dpn*II. The efficiency of RDA was measured by differential probing of Southern-blotted cloned fragments with ^{32}P-labelled BM−N and BM+N cDNA. Of more than 300 clones analysed, about 200 were potentially degreening-specific or enhanced compared to the BM+N control. To further test the expression of these 200 clones, BM−N- and BM+N-amplified cDNAs were size-fractionated on a gel, blotted and probed with digoxigenin-labelled DNA from individual clones. Figure 5 shows examples of this screening. Most of the clones analysed were more abundant in BM−N degreening cells (e.g. clones 8, 138 and 188), and a small number of clones were specific to degreening cells (clones 4, 10). A total of 41 clones which were either specific to, or more abundant in, BM−N cultures were further analysed.

Analysis of subtracted clones

RDA fragments and plaques from differential screening were sequenced and all sequences compared to each other to indicate relative abundance (Table 1). The most abundant clone, *dee*4 (for degreening-enhanced expression), represented about 42% of all investigated clones and was present in both RDA and differentially screened clones. A total of 13 independent sequences were obtained and subsequently

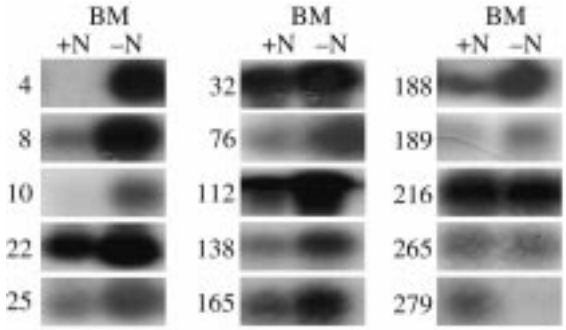

Figure 5. Screening of subtracted clones. BM+N- and BM−N-amplified cDNAs were electrophoretically separated and hybridized with individual, digoxigenin-labelled subtracted clones as numbered.

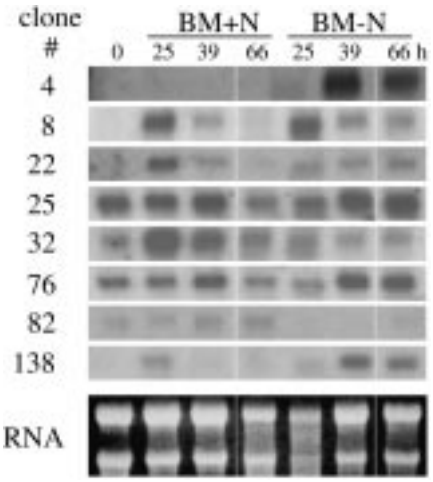

Figure 6. Expression of subtracted, non-redundant cDNA clones in *Chlorella protothecoides*. Total RNA was isolated after 0, 25, 39 and 66 h growth in either BM+N or BM−N. Part of the ethidium bromide-stained gel containing ribosomal RNAs is depicted in the bottom panel.

analysed on northern blots of total RNA isolated from BM−N and BM+N cultures (Figure 6). Four different expression patterns were obtained: (1) exclusive expression during degreening (clone 4), (2) enhanced expression at later stages of degreening (8, 22, 138), (3) as 2 but also expression in green cells (25, 76), and (4) non-specific expression (32, 82). Probes from clones 10, 112, 165, 188 and 189 did not result in a detectable signal on northern blots. Since these clones are more abundant in BM–N cDNA compared to BM+N cDNA (Figure 5) it is concluded that they are also up-regulated during degreening. When amplified cDNA from autotrophically grown cells was used to probe these clones, no hybridization was observed (data not shown) indicating that they belong to group 2.

Since the cDNA fragments obtained by RDA were rather short (200–300 bp), independent clones with expression patterns 1, 2 or 3 were used to screen a cDNA library of degreening *C. protothecoides*. In each case, the plaque containing the longest insert was given a *dee* number and the sequence compared to DNA and protein databases using the program BLAST (Table 1). The only degreening-specific clone identified, *dee*4, contains a partial open reading frame of 830 bp coding for an amino acid carrier. This putative *Chlorella* protein contains a unique domain and, to our knowledge, is distinct from all known plant amino acid transporters (Figure 7). The sequences of *dee*22 and *dee*76 show significant homology to a cell division factor (Mine) of *Synechocystis* (Kaneko *et al*., 1995) and to MO25 of mice (Miyamoto *et al*., 1993), respectively. These two putative proteins, together with *dee* 189 showing homology to extensin-like proteins, are likely to play a role in cell development. Other clones such as *dee*10 and *dee*165 were homologous to metabolic proteins and probably code for ubiquinone-cytochrome *c*-reductase and ATP-dependent citrate lyase, respectively. In addition, putative sequences for *Chlorella* ferritin (*dee*188) and for a Ca^{2+}-binding protein (*dee*112) were identified. *Dee*s 8, 25 and 138 showed no significant homology to sequences in the DNA or protein databases. Comparison of the putative open reading frames of cDNAs to the transcript size on northern blots (Figure 6) and the results of the database search indicates that full-length clones have been obtained for *dee*s 10, 22 and 76 and probably for *dee*8.

Discussion

Characterization of degreening in Chlorella protothecoides

Degradation of Chl comparable to the visual degreening observed during leaf senescence and fruit ripening in higher plants can be induced in *Chlorella protothecoides* by nitrogen deprivation. From the isolation of intermediary and final catabolites, and the characterization of catabolic enzymes, the biochemical mechanism of Chl breakdown in higher plants could be established recently (for a review, see Matile *et al*., 1999). If a senescing plant is treated with chelators such as *o*-phenanthroline or BIP, artificial

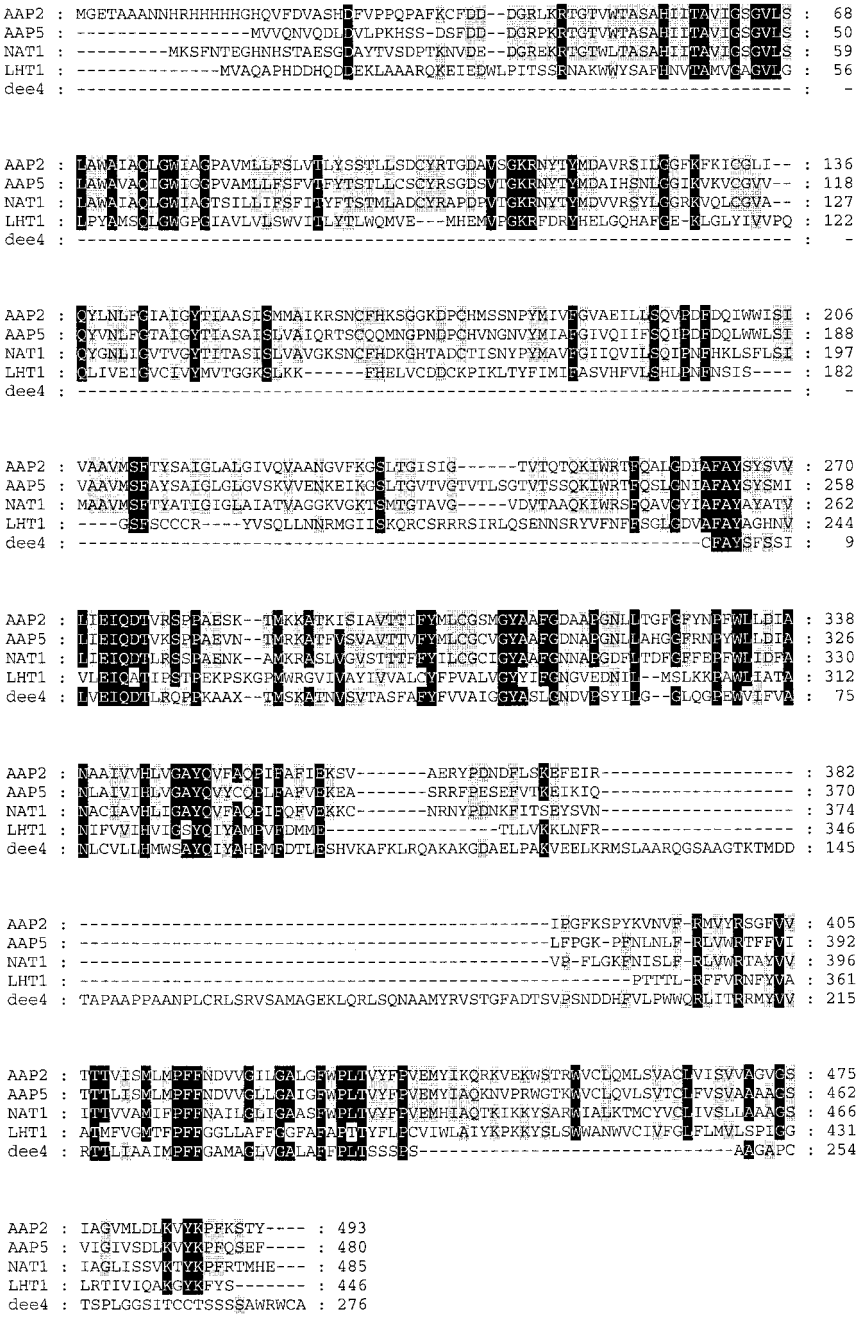

Figure 7. Alignment of the protein sequence derived from the partial cDNA sequence of *dee*4 with *Arabidopsis thaliana* amino acid carrier sequences from the GenBank/EMBL databases. AAP2, amino acid permease 2 (Kwart *et al*., 1993); AAP5, amino acid permease 5 (Fisher *et al*., 1995); NAT2, neutral amino acid transporter 2 (Hsu *et al*., 1993); LHT1, lysine-histidine-specific amino acid transporter 1 (Chen and Bush, 1997). Filled boxes indicate residues present in at least four of the five sequences, whereas residues are shaded when present in at least three sequences.

Table 1. Characterization of degreening-enhanced (*dee*s) cDNA clones from *Chlorella protothecoides.*

Chlorella clone	Number of clones[a]	Insert size (bp)	Transcript size (kb)	Expression pattern[b]	Accession No.	Putative identity	Related sequence (Accession number)[c]	% Identity/ similarity[d]
*dee*4	28 (9/19)	1216	2.1	1	AJ238635	Amino acid carrier	*Arabidopsis* (Q39136)	38/65
*dee*8	11 (10/1)	907	0.9	2	AJ238625	unknown		
*dee*10	1 (0/1)	313			AJ238626	Ubiquinone-cyt *c*-reductase	Potato (P46270)	48/64
*dee*22	1 (1/0)	917	0.8	2	AJ238631	Mine protein	*Synechocystis* (Q55899)	28/59
*dee*25	5 (5/0)	1308	0.7	3	AJ238634	unknown		
*dee*76	1 (1/0)	1466	1.3	3	AJ238632	MO25	Mouse (Q06138)	51/72
*dee*112	3 (2/1)	938			AJ238627	Ca^{2+}-binding protein	*Arabidopsis* (O22788)	35/58
*dee*138	1 (1/0)	850	1.0	2	AJ238630	unknown		
*dee*165	1 (1/0)	745			AJ238633	ATP-dependent citrate lyase	Fission yeast (P78844)	61/72
*dee*188	3 (1/2)	1165			AJ238628	Ferritin	Cowpea (Q41709)	50/67
*dee*189	1 (1/0)	284			AJ238629	Extensin-like protein	Tobacco (Q08195)	42/42

[a]The values in parenthesis indicate the number of clones obtained by RDA and differential screening, respectively.
[b]See details on the different expression patterns in Results.
[c]Most similar sequence as identified by BLASTX search (GCG).
[d]Identity/similarity based on protein comparison with the program BESTFIT in GCG 8.1 using the default values.

stay-green phenotypes can be produced (Langmeier *et al*., 1993). The stay-green effect is caused by the inhibition of Pheide *a* oxygenase responsible for porphyrin macrocycle cleavage during Chl breakdown (Hörtensteiner *et al*., 1998). The addition of BIP to degreening *C. protothecoides* cultures had the same effects as in higher plants: it inhibited Chl breakdown and delayed protein degradation. In both *Chlorella* and oilseed rape, porphyrin macrocycle cleavage is catalysed by a monooxygenase (Curty *et al*., 1995; Hörtensteiner *et al*., 1998), and so the algal enzyme probably contains iron, like its higher-plant equivalent (Hörtensteiner *et al*., 1995). Therefore, the inhibition of Chl breakdown in *Chlorella* in the presence of BIP is probably due to the inactivation of the monooxygenase as well. However, in contrast to higher plants, treatment with BIP did not cause the accumulation of the catabolites produced upstream of macrocycle cleavage, Chlide and Pheide *a*. This raises the question, what is the exact substrate of porphyrin cleavage in the alga?

In higher plants, cycloheximide and other inhibitors of cytoplasmic protein biosynthesis prevent senescence (Thomas *et al*., 1989), whereas chloramphenicol, an inhibitor of plastid-located protein synthesis, has no such effect (Thomas and Stoddart, 1980). Corresponding results were observed in degreening *Chlorella*, indicating that nuclear-encoded proteins are indispensable for Chl catabolism and efficient protein breakdown in the alga. Protein degradation during degreening not only depended on new protein biosynthesis, as demonstrated by the accumulation of cysteine protease-like proteins and induction of protease activity (Figure 4), but could also be delayed when nitrogen was available in the culture medium (BM+N). This effect was obvious when the fate of individual proteins under nitrogen-rich or nitrogen-deficient conditions was investigated on western blots (Figure 3). Degradation of components of the photosynthetic apparatus (LHCII and Cyt *f*) in BM−N was in agreement with earlier investigations with *C. protothecoides*, in which photosynthetic activity was found to decrease in glucose-containing medium (Oshio and Hase, 1972). When nitrogen was not limiting, bile pigment formation was completely abolished, although Chl and proteins were degraded in substantial amounts (Figure 2). The fate of Chl under these conditions remains elusive. However, it is notable that a colourless pigment has previously been identified as a minor Chl catabolite during degreening in *Chlorella* under nitrogen deprivation (Engel *et al*., 1996) which might occur in the presence of nitrogen as well. Overall, the results presented here suggest that the process of degreening in *Chlorella* is comparable to Chl catabolism during senescence in higher plants.

Degreening-related genes

Differential and subtractive screenings have been shown to be valuable methods for the isolation of genes that are specifically expressed in a particular environment or at a specific stage of development. Thus, a number of senescence-related genes have previously

been isolated from senescing leaves and ripening fruits (Buchanan-Wollaston, 1997). However, a major drawback in the systems investigated so far is the lack of synchrony of senescence in the material sampled because of the heterogeneity of the tissue. A unicellular green organism such as *C. prototheco*ides could, therefore, make an ideal system in which to study senescence (and hence Chl catabolism) at the molecular level. In addition to a differential screen, a subtractive method (RDA) was also used to identify less abundant genes (Buchanan-Wollaston and Ainsworth, 1997). This approach was justified, as 6 of the 11 degreening-enhanced clones were identified by RDA only. Of these 11 cDNAs, only *dee*4, which codes for a putative amino acid carrier, was exclusively expressed under BM−N conditions. Thus, the *dee*4 gene product most probably is involved in remobilization of amino acids accumulating during protein degradation. Both specificity and location of the carrier remain elusive, but homology to other amino acid carriers indicates that it could be located in the plasmalemma (Kwart *et al*., 1993). The expression patterns of the other *dee*s isolated are difficult to interpret, although in all clones expression was greater in later stages of degreening. This is reasonable since the BM−N and BM+N cDNAs used for screening the clones were derived from total RNA isolated after 50 h of incubation. Thus, in addition to a role in green cells (class 3 clones), these genes seem to be important during later stages of degreening in *Chlorella*. Notably, genes for ferritin (*dee*188) and ATP-dependent citrate lyase (*dee*165) have previously been identified as being senescence-enhanced in rape (Buchanan-Wollaston *et al*., 1997) and banana peel (R. Drury and G.B. Seymour, unpublished), respectively. The role of these proteins in senescence is unclear, but their identification in both *Chlorella* and higher plants emphasizes the similarity of the degreening processes in these phylogenetically distinct systems.

The purification and isolation of cDNAs encoding proteins that are involved in Chl breakdown has, with one recent exception, not been successful. The notable exception is that of a cDNA from *Citrus* which when expressed in *Escherichia coli* resulted in chlorophyllase activity (E.E. Goldschmidt, personal communication). Purification of other Chl-degrading proteins from higher plants has, at least for Pheide *a* oxygenase, proved to be problematic (K.L. Wüthrich and S. Hörtensteiner, unpublished). In addition, purification of the analogous protein(s) from *Chlorella prototheco*ides most probably may be impossible because such algae are known to be unsuitable for preparation of intact chloroplasts or gentle extraction of proteins due to tough cell walls.

However, none of the screening attempts made in a number of species and different senescing tissues (Buchanan-Wollaston, 1997), including the present one, have as yet identified a gene that is directly involved in Chl degradation. A major problem is that no related enzymes from other systems have been identified. For example, comparison of porphyrin ring cleavage of haem by haem oxygenase (Tenhunen *et al*., 1969) and of Pheide *a* by Pheide *a* oxygenase (Hörtensteiner *et al*., 1998) suggests that the mechanisms are completely different. It is unlikely, therefore, that the two proteins share sequence similarity. In addition, a Chl catabolic function of anonymous cDNAs could only be assigned by the production of transgenics, knockout mutants or by heterologous expression.

The chances of isolating genes of Chl degradation in our opinion can significantly be increased by differential methods when employing a simple plant system whose metabolism can easily been modified. We are confident that *Chlorella prototheco*ides is suitable for this purpose.

Acknowledgements

This work was supported by the Swiss National Science Foundation. The Institute of Grassland and Environmental Research is sponsored by the UK Biotechnology and Biological Science Research Council. We thank Greg Amstrong, ETH Zürich, Switzerland for providing the *Arabidopsis* POR antibody.

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