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The purification of the *Chlamydomonas reinhardtii* chloroplast ClpP complex: additional subunits and structural features

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Abstract The ClpP peptidase is a major constituent of the proteolytic machinery of bacteria and organelles. The chloroplast ClpP complex is unusual, in that it associates a large number of subunits, one of which (ClpP1) is encoded in the chloroplast, the others in the nucleus. The complexity of these large hetero-oligomeric complexes has been a major difficulty in their overproduction and biochemical characterization. In this paper, we describe the purification of native chloroplast ClpP complex from the green alga *Chlamydomonas reinhardtii*, using a strain that carries the Strep-tag II at the C-terminus of the ClpP1 subunit. Similar to land plants, the algal complex comprises active and inactive subunits (3 ClpP and 5 ClpR, respectively). Evidence is presented that a sub-complex can be produced by dissociation, comprising ClpP1 and ClpR1, 2, 3 and 4, similar to the ClpR-ring described in land plants. Our *Chlamydomonas* ClpP preparation also contains two ClpT subunits, ClpT3 and ClpT4, which like the land plant ClpT1 and ClpT2 show 2 Clp-N domains. ClpTs are believed to function in substrate binding and/or assembly of the two heptameric rings. Phylogenetic analysis indicates that ClpT subunits have appeared independently in Chlorophycean algae, in land plants and in dispersed cyanobacterial genomes. Negative staining electron microscopy shows that the *Chlamydomonas* complex retains the barrel-like shape of homo-oligomeric ClpPs, with 4 additional peripheral masses that we speculate represent either the additional IS1 domain of ClpP1 (a feature unique to algae) or ClpTs or extensions of ClpR subunits.

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Abbreviations

ACN	Acetonitrile
ADEP	Acyldepsipeptide
MS	Mass spectrometry

Introduction

Clp proteases are ubiquitous self-compartmentalized serine proteases that associate a ClpP complex carrying peptidase activity with AAA+ chaperones from the Clp/HSP100 family. They arose from bacterial origins and are found in the mitochondria and chloroplasts of eukaryotic cells as a consequence of the endosymbiotic origin of these organelles. The structure and function of ClpP peptidases has been extensively studied in bacteria especially in *E. coli* where it was identified for the first time (Hwang et al. 1987; Katayama-Fujimura et al. 1987). Today, X-ray crystallographic structures of ClpP complexes are available for six bacteria (*Escherichia coli*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Helicobacter pylori, Bacillus subtilis, Staphylococcus aureus*) and for human mitochondria (Bewley et al. 2006; Gribun et al. 2005; Ingvarsson et al. 2007; Kang et al. 2004; Kim and Kim 2008; Lee et al. 2011; Zhang et al. 2011). All show a barrel-shaped structure formed by two closely apposed rings, each composed of seven identical ClpP subunits. The residues of the catalytic triad (Ser, His, Asp,) line an inner cavity where proteolysis occurs. Substrates unfolded by the hexameric chaperones bound to the apical surfaces of the ClpP barrel are fed into this chamber in an extended conformation and cleaved into short peptides in a processive manner.

While most bacteria possess a unique ClpP gene, some have been found to harbor two (e.g. *Mycobacterium tuberculosis*), three (*Agrobacterium tumefaciens*) or even four (most Cyanobacteria) ClpP-type genes (Yu and Houry 2007). The physiological form of *M. tuberculosis* ClpP might actually be a tetradecamer composed of homomeric heptamers of ClpP1 and ClpP2 (Geiger et al. 2011).The Cyanobacterium *Synechococcus elongatus* carries 3 ClpP genes (*clpP1*, *clpP2* and *clpP3*), plus a *clpR* gene, encoding a subunit deemed inactive because it lacks critical residues of the catalytic triad. These four proteins are associated into two distinct complexes: a ClpP1/ClpP2 complex and a ClpP3/ClpR complex (Stanne et al. 2007). The former resembles more the proteobacterial ClpP, which has given rise to the mitochondrial complex. In plants and algae, mitochondrial ClpP is formed of a single subunit named ClpP2 (Adam et al. 2001). In contrast, the cyanobacterial ClpP3/ClpR complex is proposed to have evolved into the chloroplast complex after endosymbiosis: the chloroplast *clpP1* and the nuclear *CLPR2* genes are clearly derived from the cyanobacterial *clpP3*, while the *clpR* gene has given rise to three nuclear genes *CLPR1*, *CLPR3* and *CLPR4* (Majeran et al. 2005). All these subunits are part of a "ClpP1/R-ring" found in land plants, believed to associate with a "ClpP" ring to form the ClpP/R core complex (Olinares et al. 2011; Sjogren et al. 2006). The ClpP ring associates active, nuclear-encoded, ClpP subunits (ClpP3, ClpP4, ClpP5 and ClpP6 in Arabidopsis), whose phylogenetic origin is uncertain, but that were clearly acquired early in the evolution of photosynthetic eukaryotes (Majeran et al. 2005). In green algae, a single ortholog exists for ClpP3 and ClpP4 (it has been called ClpP4), while ClpP6 has undergone mutations that render it presumably inactive, hence the name ClpR6 for the algal ortholog.

In addition, new, unrelated subunits have been found in the Arabidopsis ClpP complex. These subunits were originally called ClpS1 and ClpS2 (Peltier et al. 2001, 2004), but are now referred to as ClpT1 and ClpT2 to avoid confusion with the more recently discovered ClpS modulator of Hsp100 chaperones, which is also present in the chloroplast (Zybailov et al. 2009). Their function remains unknown even though based on 3D models of both ClpT1, and ClpT2 and the ClpP/R complex, Peltier et al. (2004) proposed a model for interaction between ClpPR core peptidase and the ClpT proteins. ClpT1 and ClpT2 are predicted to bind on an apical side of the ClpP/R complex through the P1 hydrophobic pockets. Based on this model, they proposed that ClpTs could be implicated in the regulation of Clp proteolytic activity by modulating docking of the ClpC chaperone and substrate delivery (Olinares et al. 2010). Recently, in vitro reconstitution experiments suggested that ClpTs are involved in assembly of the ClpP/R core complex, with ClpT1 first binding to the P-ring followed by ClpT2 binding and formation of the core (Sjogren and Clarke 2011).

Chloroplast ClpP is a central component of the chloroplast proteolytic network, and most of its subunits are essential in *Chlamydomonas* and/or land plants (Huang et al. 1994; Kim et al. 2009; Kuroda and Maliga 2003; Zheng et al. 2006). Yet the complexity of its organization and its relative low abundance make its biochemical study difficult. As a consequence the biochemical data available on chloroplast ClpP have been obtained mostly by separation of chloroplast stroma by native electrophoresis, followed by antibody staining or two-dimensional electrophoresis and mass spectrometry (MS). But the use of in vivo affinity-tagging techniques for the purification of the ClpP/R complex clearly opens new perspectives for its

study. These techniques have allowed the dissection of the complex processing pathway of the unusual *C. reinhardtii* ClpP1 (Derrien et al. 2009), as well as the purification of the ClpP/R complex of *A. thaliana* and the determination of its subunit stoichiometry within each heptameric ring and the intact core complex (Olinares et al. 2011). In this study, we describe a method to purify native and active chloroplast ClpPR complex from the green alga *Chlamydomonas reinhardtii* and present the initial characterization of the complex.

Experimental procedures

ClpP complex purification-*Chlamydomonas reinhardtii*

The ClpP1-Strep strain construction has been described previously in Derrien et al. (2009), supplemental methods. Strain ClpP1-TEV-Strep was obtained by inserting the sequence gatattccaactactgctagtgagaatttgtattttcagggt encoding the TEV-protease cleavage site between the NheI and SpeI sites of pClpP1-Strep. Cells were grown in Tris acetate/phosphate medium under constant illumination (40 µE m^{-2} s^{-1}) at 25 °C, in 5 L stirred Erlenmeyer flasks, or 10 L carboys with air bubbling. Cells were collected at the end of exponential growth phase (6.10^6 cells per mL) and washed once in Tris–HCl 20 mM pH 8.0, NaCl 150 mM (Buffer A). The final volume was adjusted to 80 mL and protease inhibitors were added (1 mM benzamidine, 5 mM amino-6-caproic acid and 1 mM EDTA). Cells were then broken using a cold French press at 385 psi and all subsequent steps were carried out at 4 °C. The lysate was centrifuged at 20,000 rpm for 30 min. After adding 5 mM $MgCl_2$ to promote thylakoid stacking (Barber and Chow 1979), the supernatant was clarified by ultracentrifugation (60,000 rpm, 1 h in a 70Ti Beckman Coulter rotor). Ammonium sulfate was added to the supernatant at 45 % saturation. After 30 min under gentle stirring, precipitated proteins were pelleted by centrifugation for 5 min at 5,000 rpm (JA-12 rotor). This served to pre-purify the complex and to remove an unidentified non-dialyzable inhibitor of the streptagII/streptactin binding reaction (not shown). The pellet was solubilized by gentle shaking on a rotary wheel for 15 min in Buffer A supplemented with 10 % glycerol to prevent partial dissociation during chromatography and the final steps of concentration and freezing. The protein extract was adjusted to 10 mL and applied to a StrepTrap HP column (GE Healthcare). Cr-ClpP complex was eluted in buffer E (B + 2.5 mM Desthiobiotine, Sigma), buffer-exchanged and concentrated in buffer B in a 100,000 MW kDa cut off Centricon, and stored at −80 °C.

Activity measurements

Cleavage of the peptide FAPHMALVPV by Cr-ClpP was carried out at 25 °C with 2 mM peptide substrate in a final volume of 25 µl of 50 mM Tris/HCl, pH 8.0 containing 0.1 M KCl. Cr-ClpP (1.8 µg) was added to start the reaction. At the indicated times, the reactions were stopped by addition of an equal volume of 8 M Guanidine/HCl, samples were loaded onto a C18-HPLC column, and the separated products were quantified by integration of the 210 nm absorbance peaks (Maurizi et al. 1994).

Denaturing electrophoresis and western blot analysis

Denaturing electrophoresis (SDS/PAGE) was carried out in 12–18 % acrylamide gradient gels containing 8 M urea (Piccioni et al. 1981). Proteins were stained with Coomassie blue R-250, or electroblotted onto nitrocellulose membrane (Hybond-ECL, GE Healthcare). Antibodies to ClpP1, ClpR2, ClpP5 and ClpP6 have been described before (Majeran et al. 2000, 2005; Zheng et al. 2002). Immunoblots were revealed with the ECL system (GE Healthcare).

Colorless-native PAGE

Protein extract were supplemented with 500 mM amino-6-caproic acid, 50 mM Bistris HCl (pH 7.4), 15 % glycerol and 0.004 % Ponceau Red. Samples were loaded onto an 18-cm long polyacrylamide 6–14 % gradient gel (Schagger et al. 1994). Electrophoresis was carried out overnight at 4 °C, at 350 V. Molecular size markers were ovalbumin (44 kDa), aldolase (158 kDa), ferritin (440 kDa) and thyroglobulin (670 kDa).

Mass spectrometry analysis

Mass spectrometry was performed either with an LTQ-Orbitrap or with an LTQ-XL ion trap. For the LTQ-Orbitrap analysis, in gel digestion was performed as described in Friso et al. (2011). All mass spectrometry data were collected using a LTQ-Orbitrap interfaced with a nanoLC system and autosampler (Thermo) using data dependent acquisition and dynamic exclusion (repeat count 2), with the Orbitrap portion operating at 100.000 resolution, as described in Friso et al. (2010, 2011). Peak lists (in.mgf format) were generated from RAW files using DTA supercharge software, and all.mgf files were recalibrated as in Friso et al. (2011). Recalibrated files were searched with MASCOT v2.2 against Chlamydomonas v4 proteins (Joint Genome Institute), including a small set of typical contaminants and the ClpP-StrepII modified sequence. Two parallel searches (Mascot p value < 0.01; precursor ion

window 700 to 3,500 Da) were carried out: (1) full tryptic (6 ppm) with variable M-ox, Gln to pyro-Glu (N-termQ), N-term protein acetylation, and fixed Cys-carbamidomethylation, 2 missed cleavages (in Mascot PR or PK does not count as missed cleavage), (2) semi-tryptic (3 ppm) with variable M-ox, N-term acetylation, Gln to pyro-Glu (N-termQ) and fixed Cys-carbamido-methylation, 2 missed cleavages.

For nanoLC-LTQ-XL analysis the in-gel digestion was performed with the Progest system (Genomic Solution) according to a standard trypsin protocol, extracted successively with 2 % trifluoroacetic acid and 50 % ACN and then with ACN. Peptide extracts were dried and suspended in 20 µL of 0.05 % trifluoroacetic acid, 0.05 % HCOOH, and 2 % ACN. On-line HPLC was performed on a NanoLC-Ultra system (Eksigent), using the same C18 pre columns and analytical columns as for the Orbitrap analysis and comparable gradients. Ionization (1.5 kV ionization potential) was performed with liquid junction and a noncoated capillary probe (10 µm i.d.; New Objective). Peptide ions were analyzed using Xcalibur 2.07 with the following data-dependent acquisition steps: (1) full MS scan (mass-to-charge ratio (m/z) 300 to 1,400, centroid mode) and (2) MS/MS (qz = 0.25, activation time = 30 ms, and collision energy = 35 %; centroid mode). Step 2 was repeated for the three major ions detected in step 1. Dynamic exclusion was set to 30 s.

A database search was performed with XTandem (version 2009.04.01.1) (http://www.thegpm.org/TANDEM/). Enzymatic cleavage was declared as a trypsin digestion with one possible missed-cleavage. Cys carboxyamidomethylation and Met oxidation were set to static and possible modifications, respectively. Precursor mass and fragment mass tolerance were 2.0 and 0.8 Da, respectively. A refinement search was added with similar parameters except that semi-tryptic peptide and possible N-terminal proteins acetylation were searched. We used the Chlamydomonas JGI v4 database (downloaded from http://genome.jgi-psf.org/Chlre4/Chlre4.home.html) supplemented with chloroplast and mitochondrial proteins (from NC_005353, NC_001638), along with a contaminant database (trypsin, keratins, …). Only peptides with an E value smaller than 0.1 were reported. Identified proteins were filtered and grouped using XTandem Pipeline (http://pappso.inra.fr/bioinfo/xtandempipeline/) according to: (1) A minimum of two different peptides was required with a E value smaller than 0.05, (2) a protein E value (calculated as the product of unique peptide E values) smaller than 10^{-4}. In case of identification with only two or three MS/MS spectra, similarity between the experimental and the theoretical MS/MS spectra was visually checked. To take redundancy into account, proteins with at least one peptide in common were grouped. This allowed to group proteins of similar function. Within each group, proteins with at least one specific peptide relatively to other members of the group were reported as sub-groups.

Electron microscopy and image analysis

For EM, purified ClpP was brought to a final concentration of 30–50 µg/mL in 50 mM Tris HCl (pH 7.5), 150 mM KCl and 10 mM $MgCl_2$. A 3.5 µl drop was applied to a carbon coated copper grid. After 30 s of absorption, excess sample was blotted away and the grid was soaked on two successive drops of uranyl acetate stain (2 %) for 20–30 s, blotted, and allowed to dry. Micrographs were recorded on a Philips CM120 electron microscope operating at 120 kV and 60,000 magnification. Micrographs were recorded on Kodak SO-163 film and developed 12 min in D19.

Drift-free micrographs were scanned on a Zeiss scanner at 14 µm step size, which gives a final pixel size of 2.33 Å/pixel. 900 particles were picked and preprocessing, which includes CTF parameter determination and correction by phase flipping, was done with the Bsoft package (Heymann and Belnap 2007). Computation of reference-free class averages was done as described previously (Effantin et al. 2009; Hierro et al. 2007). Picked particles were first centered using EMAN (Ludtke et al. 1999). An initial global average was first generated by a reference-free approach implemented in SPIDER (Frank et al. 1996). The particle images were then aligned against the global average and classified by multi-variate statistical analysis, hierarchical ascendant clustering, and averaged to generate a first set of class averages. These initial class averages were then refined by doing more iterations of multi reference alignment, dimension reduction by correspondence analysis, hierarchical ascendant clustering, and averaging until a stable set of classes was obtained from one cycle to the next.

Results

Purification of the ClpP complex

In order to purify the *Chlamydomonas* chloroplast ClpP complex (Cr-ClpP), we have introduced a strep-tagII at the C-terminal end of the chloroplast-encoded ClpP1 subunit using chloroplast transformation. We chose this position rather than the N-terminus of the protein, because the latter contains a highly conserved sequence, MPIGV…, found in almost all ClpP1 and in the related ClpR2 proteins. We present results obtained with a tag placed immediately at the C-terminus, but similar results were obtained when a short linker sequence corresponding to the TEV-protease cleavage site was introduced upstream of the tag (data not shown). The protein was followed at each step of the

purification process using antibodies directed against ClpP1 and ClpR2 (Fig. 1). While the latter recognizes a single band, our previous work has shown that ClpP1 is proteolytically processed at 3 positions, yielding an N-terminal ClpP1 $_N$ fragment and two C-terminal fragments of lesser immunoreactivity, ClpP1$_C$ and ClpP1$_{C'}$, in addition to the ClpP1$_H$ precursor (Derrien et al. 2009). All bands showed the same behavior during purification, indicating their tight association.

Peptidase activity of Cr-ClpP

The purified complex was found capable of cleaving the model decapeptide FAPHMALVPV, derived from the N-terminal part of *E. coli* ClpP and spanning the propeptide cleavage site between the methionine and alanine residues (Thompson et al. 1994). Cleavage was followed by HPLC (Maurizi et al. 1994) and yielded products identical to those generated by the *E. coli* complex (Ec-ClpP), although the activity was substantially lower (~7.8 nmol/min/nmol complex, which is 0.8–1 % of that of Ec-ClpP under comparable conditions). Cleavage was not activated by the addition of either Ec-ClpA or Ec-ClpX. Surprisingly, cleavage was also not activated by the acyldepsipeptide (ADEP) which allosterically opens the axial channel of bacterial ClpPs and makes the degradation chamber highly accessible to peptides and unfolded proteins (Brotz-Oesterhelt et al. 2005; Li et al. 2010). In contrast, the peptidase activity of Cr-ClpP was inhibited by ADEP (data not shown), indicating that ADEP does interact with Cr-ClpP but promotes a different conformational change. Cr-ClpP did not cleave the fluorogenic dipeptide Suc-Leu-Tyr-AMC which is an another substrate for Ec-ClpP (Thompson and Maurizi 1994). Freeze-thawing of the preparation or addition of small amounts of detergent (Triton X-100, NP-40) did not lead to Suc-Leu-Tyr-AMC cleavage. These results suggest that the axial channel of the chloroplast enzyme is not constitutively open to short peptides or that the active site cannot accommodate the bulky coumarin moiety.

Identification of subunits using MS/MS analysis and Edman sequencing

Analysis of the Cr-ClpP preparation by non-denaturing Colorless-Native electrophoresis (CN-PAGE) and immunoblotting with the ClpR2 antibody (Fig. 2, left panel) revealed that the purified ClpP migrates as a sharp band at 540 kDa, the same size as the native complex observed in stromal extracts. In addition, a diffuse smear was observed in the high MW range, as well as faint diffuse bands between 300 and 440 kDa. The high MW smear probably represents aggregated forms generated during complex preparation or electrophoresis. Similarly, the lower MW immuno-reactive material could result from dissociation, but the presence in the French press lysate of a faint band around 350 kDa (lane 1; see also Figs. 4 and 5 in ref Majeran et al. 2005) rather suggests that it corresponds to the ClpP1/R ring previously described in Arabidopsis extracts (Olinares et al. 2011). Efforts to dissociate the purified complex by freeze-thawing were unsuccessful (lane 4). Dissociation was also not observed when the salt concentration was raised during cell lysis or, as described for Ec-ClpP (Maurizi et al. 1998), when the purified complex was incubated with potassium sulfate (not shown). However, dissociation could be obtained by omitting glycerol from the preparation buffers (Fig. 2, lanes 5–6), in which case the sharp 540 kDa band was reduced or absent, and the low MW form predominated, sometimes accompanied by a high MW band around 670 kDa.

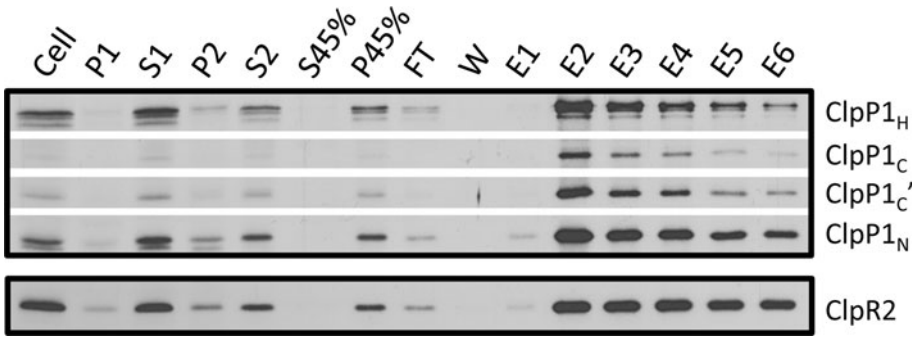

Fig. 1 Western blot analysis of ClpP complex purification, using antibodies against ClpP1 (*top*) and ClpR2 (*bottom*). Gel loading was 10 µg chlorophyll for the total cell extract and an equivalent volume for the other fractions: pellet (P) and supernatant (S) of the 1 st and 2nd centrifugations and of the 45 % ammonium sulfate precipitation; flow-through (FT) and wash (W) of the affinity column. For fractions eluted from the column (E1 to E6, successive applications of elution buffer), a larger fraction of the sample was loaded, corresponding to 1/1,000 of the entire preparation

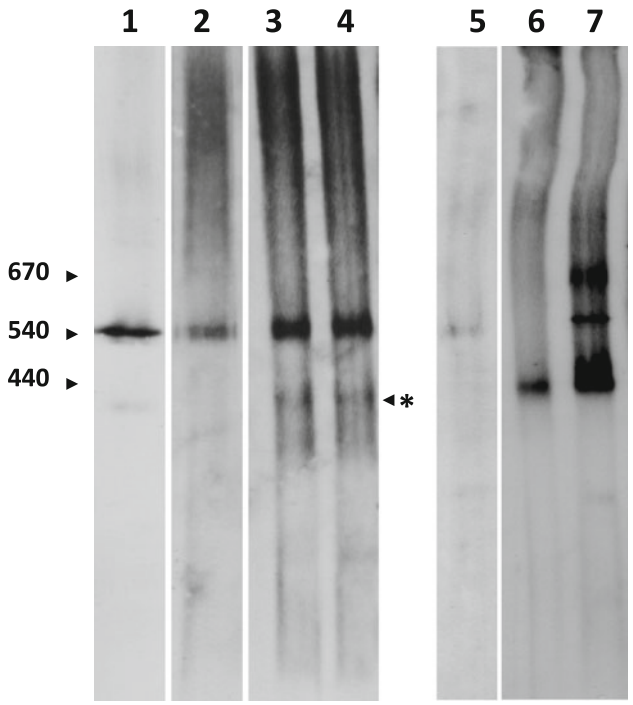

Fig. 2 Native gel electrophoresis of the ClpP complex: Immunoblots reacted with the ClpR2 antibody. *Left* and *right* panel are from different immunoblots. (*1*) soluble cell extract; (*2*) eluate from Streptactin column; (*3*) final ClpP preparation after concentration on the Vivaspin® column; (*4*) same as 3, after one cycle of freezing/thawing. The *asterisk* indicates the position of the low molecular weight band that is also observed in whole cell extracts and that has been identified as the ClpP1/R ring by mass spectrometry. (*5*) purified Cr-ClpP; (*6*) and (*7*) ClpP complex purified from the ClpP1-strep and ClpP1-TEV-Strep strains, respectively, according to the standard protocol except that glycerol was omitted from all buffers; all samples subjected to one cycle of freeze-thawing. Note dramatic increase of the low MW band

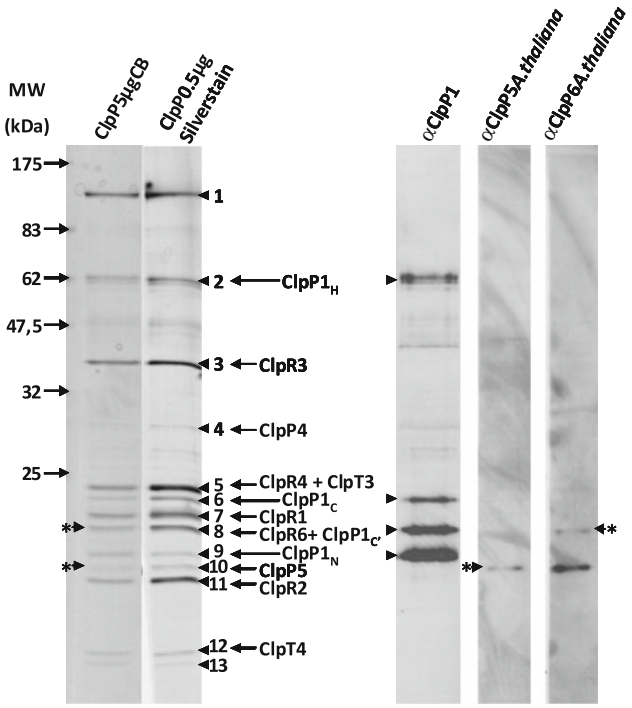

Fig. 3 Analysis of the ClpP complex using denaturing gel electrophoresis. *Left* Coomassie blue and silver nitrate staining of a typical preparation. The bands numbered have been analyzed by MS/MS or Edman degradation (see Table 1) *Right* immunoblots of the complex with antibodies to *Chlamydomonas* ClpP1 and to Arabidopsis ClpP5 and ClpP6 peptides (antigens identified by *asterisk*)

Denaturing SDS-PAGE of Cr-ClpP revealed 13 major bands, plus a few contaminating bands whose intensity varied from one preparation to another (Fig. 3; Table 1). This is more than the number of known ClpP/R genes. This is in part because the ClpP1 subunit of *Chlamydomonas* differs from that of land plants, in presenting a large additional domain, the "insertion sequence" (IS1) that is proteolytically matured at three distinct cleavage sites inside IS1 (Derrien et al. 2009). Consequently, the chloroplast *clpP1* gene contributes four different products, which were all identified in our Cr-ClpP preparation. Mass spectrometry and Edman degradation data, part of which has been reported in Derrien et al. (2009), identified, all ClpP/R proteins known from the *C. reinhardtii* genome sequence, except for the mitochondrial ClpP2 (Table 1, see details in Supplemental Fig. 1 and Table 1). This confirms and extends findings of our previous study of *Chlamydomonas* ClpP, carried out on crude stromal extracts (Majeran et al. 2005).

The band corresponding to $ClpP1_H$, the unprocessed clpP1 gene product, (around 60 kDa, band 2) was always found as a doublet, a characteristic of Strep-tagged ClpP1 which was not found when other tags were used (Derrien et al. 2009). The second largest subunit and the most intensely stained by Coomassie blue and silver nitrate was identified as ClpR3. ClpP4 was found in a faint band just below, and its N-terminal sequence indicated a chloroplast targeting peptide (cTP) of 49 residues. It was followed by a group of four closely spaced bands around 25 kDa. The largest of these comprised ClpR4 (cTP 42 AA), in a mixture with a novel subunit, which we called ClpT3 (see below). Just below was $ClpP1_C$, followed by ClpR1 and $ClpP1_{C'}$, the latter mixed with traces of ClpR6. $ClpP1_C$ and $ClpP1_{C'}$ are the two alternative products of cleavage events occurring near the end of the IS1 domain (Derrien et al. 2009). N-terminal sequencing indicated that ClpR1 lacked 164 AA at the N-terminus, much more than the length of its predicted cTP. This suggests that this protein could be processed at the N-terminus after it is imported into the stroma. The N-terminal fragment produced by ClpP1 processing, $ClpP1_N$, was found below $ClpP1_{C'}$. Below we found, ClpP5, then ClpR2. The acetyl-CoA biotin carboxyl carrier protein was often found comigrating with ClpP5,

Table 1 *C. reinhardtii* chloroplast ClpP subunits

Protein	Corresponding band in SDS-PAGE[b]	JGI protein ID	GenBank	MS/MS analysis of the bands from SDS-PAGE		MS/MS analysis of the bands from CN-PAGE		Edman sequencing	Predicted MW (kDa)			Apparent MW (kDa)
				SPC	Coverage (%)	SPC core complex	SPC ClpP1/R ring	Mature N-terminal sequence	Precursor	TargetP[c]	Mature[d]	
$ClpP1_H$	2	#	NP_958364	17	59	10	12	PIGV	60.8	#	60.6	52
$ClpP1_C$	6	#		8	48			NYLD	#	#	24.4	24
$ClpP1_{C'}$	8	#		9	73			YRK	#	#	22.7	23
$ClpP1_N$	9	#		8	54			PIGV	#	#	19.7	22
ClpP4[a]	4	182996	EDP05224	17	46	14	0	NSQ	38.4	36.8	33.2	28
ClpP5[a]	10	55336	EDP05337	22	71	6	0		27.8	25.4	#	22
ClpR1	7	183767	#	13	49	8	10	AYGD	46.2	39	28.1	24
ClpR2	11	183329	EDP04400	20	56	3	6		31.6	26.5	#	21
ClpR3	3	205482	EDP05878	25	66	15	21		46.9	42.7	#	34
ClpR4	5	147520	EDP03219	15	55	5	11	RKL	32.7	29.6	28.3	25
ClpR6	8	128308	EDP05174	5	33	10	0		31.2	27.1	#	23
ClpT3	5	195805	EDP06586	9	61	0	3		31.4	25.2	#	25
ClpT4	12	175283	EDP01528	12	77	3	5	ATATAAP	26.6	18.3	19.8	18

SPC spectral counts. #: not applicable

[a] Results shown for ClpP4 and ClpP5 in the row "MS/MS analysis of the bands from SDS-PAGE come from an independent experiment (see supplemental data, Experiment 2 Q-TOF)"

[b] Numbers refer to bands shown in Fig. 3

[c] Predicted molecular weights were calculated after removal of transit peptides given by TargetP

[d] Mature molecular weights were calculated according to mature N-terminal sequence given by Edman sequencing experiment

but we believe that it represents a contaminant of the affinity purification, binding to the column through its biotin moiety. Another new subunit, ClpT4, migrated around 17 kDa. The lowest band (#13) contained Cre05.g241150, an unknown protein with a DUF2322 domain, not related to known Clp proteins but always present in our preparations (including TAP-tagged purified ClpP). TargetP (Emanuelsson et al. 2000) predicts it as chloroplast-targeted, and MS/MS coverage was high (68 % if one considers only the predicted mature protein). Outside of *Chlamydomonas* (where a paralog was found nearby on the genome, Cre05.g241000) and Volvox, its closest homologs were bacterial proteins annotated either as UDP-N-acetylenolpyruvoylglucosamine reductase or as RNA polymerase factor sigma-32. Another protein also found in strep-tagII as well as in TAP-tag preparations was Cre16.g651650 (MTT1, mitochondrial translation termination factor-like protein, in Band 1). The association of these proteins with the ClpP/R complex deserves further study.

The exact stoichiometry of the various subunits in the complex cannot be deduced from our MS data alone. The ClpP4 and ClpP5 bands stained with a lower intensity than the others. Yet, the presence of ClpP5 was unambiguously supported by MS/MS data and western blot analysis with an antibody directed against an Arabidopsis ClpP5 peptide (Fig. 3). Of all Clp proteins, ClpR6 gave the lowest peptide count (5) and sequence coverage (33 %), and it was found only in a mixed band with $ClpP1_C$. A reaction with an antibody to the full-length Arabidopsis ClpP6 was observed in the position of the orthologuous ClpR6, but staining was stronger in the position of ClpP5, so it is possible that this subunit is not present in all complexes.

MS/MS analysis was also carried out on silver-stained native gels of the purified complex. The main, upper band yielded peptides from all the above-mentioned subunits except for ClpT3 but including Cre05.g241150 (Table 1). In addition, a lower band of lesser abundance, presumably corresponding to the one identified by immunoblotting with ClpP1 (Majeran et al. 2005) and ClpR2 (Fig. 2), was also analyzed and found to contain exclusively the subunits of the ClpP1/R ring (i.e. ClpP1, ClpR1, ClpR2, ClpR3 and ClpR4), along with ClpT3 and ClpT4. No band was seen that would correspond to a ClpP ring containing ClpP4, ClpP5 and ClpR6.

Sequence analysis of the ClpT subunits

The two new subunits described in this study, ClpT3 and ClpT4, were consistently observed with high sequence

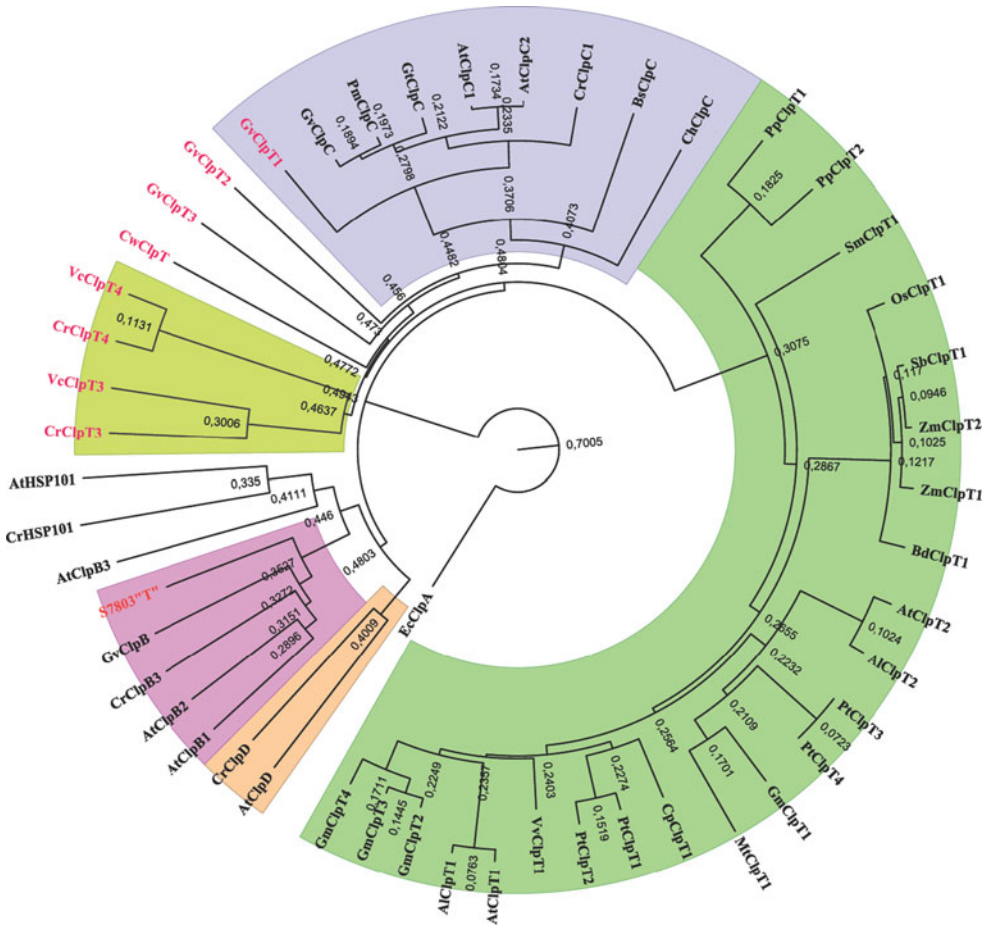

Fig. 4 Phylogenetic tree of ClpT and Clp-N domains of selected Hsp100 chaperones. The alignment shown in Suppl Fig 2 was truncated to the Clp-N domains and the tree was computed using the PHYLIP package, using the Gonnet 250 matrix, and UPGMA for the guide tree. The tree is artificially rooted at ClpA. The age of the nodes is indicated. ClpT sequences of green algae and Cyanobacteria (names labeled in *red*) cluster independently form each other and from the land plant sequences (region highlighted in *dark green*)

coverage (>60 %) in all our preparations, including in those obtained with the TAP-tag method (data not shown). These proteins had not been identified as ClpT homologs in previous surveys of the genome, but in BLAST searches, both proteins hit the N-terminal part of Clp/Hsp100 chaperones. Detailed analyses using the Superfamilly server (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/index.html) (Gough et al. 2001) revealed that ClpT3 and ClpT4 each contain two Clp-N domains, like the plant ClpT. A pair of Clp-N domains is found at the N-terminus of most Clp/Hsp100 chaperones, and they play a role in substrate recognition (Hinnerwisch et al. 2005; Kojetin et al. 2009; Ortega et al. 2000; Singh et al. 2001; Tanaka et al. 2006). Figure 4 shows a phylogenetic tree based on the alignment, presented in suppl Fig. 2, of various ClpT proteins and of the Clp-N domains of selected Hsp100 chaperones. The ClpT proteins of *Chlamydomonas* and of the related alga *Volvox carteri* do not cluster with those of higher plants. This is why, to avoid mistaking them for orthologs of the Arabidopsis ClpT1/T2, the algal proteins were named ClpT3 and ClpT4. ClpT3 was the most divergent, and had a long C-terminal extension with no similarity in the databases. Surprisingly, no ClpT gene could be identified in Chlorophyte green algae other than *Chlamydomonas* and *Volvox*. In *Chlorella*, *Coccomyxa*, *Micromonas* and *Ostreococcus* (7 genomes total), Clp-N domains were found exclusively in Hsp100 chaperones, never as a separate protein. No ClpT could be found in red algae or diatoms either.

In contrast, based on the Phytozome database (http://www.phytozome.net, cluster 12845609), most of the land plant genomes were found to contain at least one ClpT gene, sometimes two (*Arabidopsis*, Maize, *Physcomitrella*) or even four (*Populus*, *Glycine*). A ClpT gene can also be identified in ESTs from *Nitella hyalina*, a Charophyte alga found near the base of the Streptophyte (land plants)

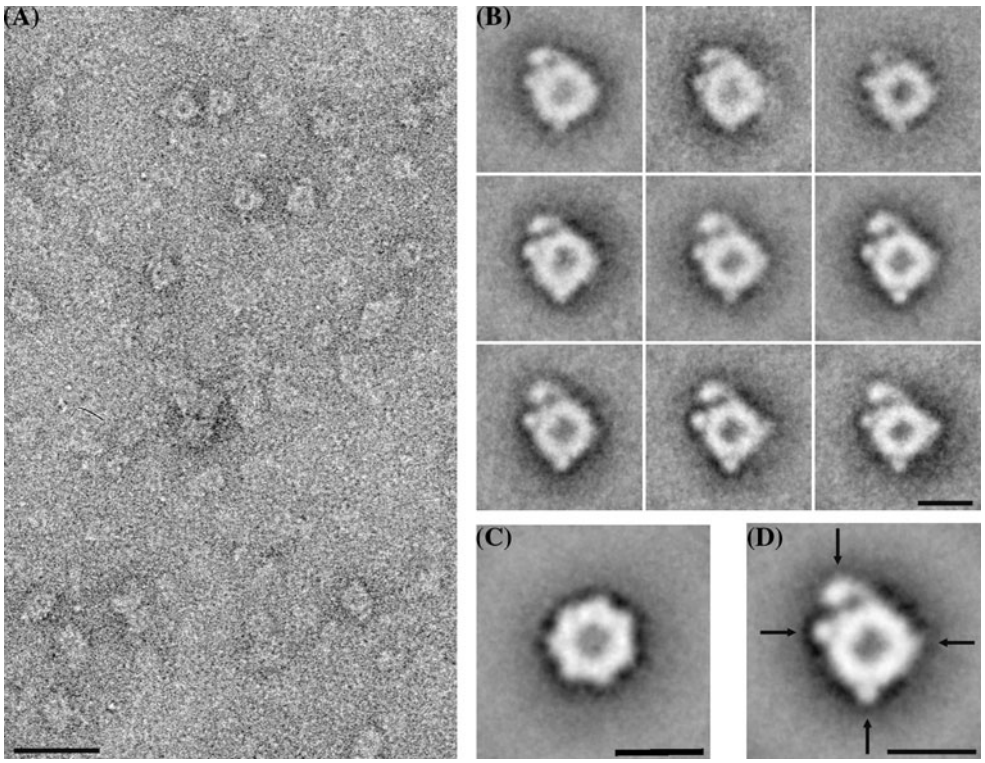

Fig. 5 Chloroplast ClpP complex retains the barrel shape structure. **a** CrClpP field of view. **b** Average image of nine classes of CrClpP particles. **c**, **d** Zoomed average images of Ec-ClpP and Cr-ClpP respectively. The four *arrows* indicate the positions of the 4 additional masses. *Scale bars* are 300 Å in **a** and 100 Å in **b–d**

lineage. It thus appears that ClpT genes have evolved twice in photosyntheic eukaryotes, first in the Streptophytes and then in the Chlorophyceae, a group of chlorophyte algae which diverged about 200–300 Myr ago, (Umen 2011).

Interestingly, two Cyanobacterial genomes also show Clp-N domains that are not part of an AAA+ chaperone. In *Crocosphaera watsonii*, CwatDRAFT_1642 contains a double Clp-N domain C-terminal to a domain conferring albicidin resistance. In *Gloeobacter violaceus*, three ClpT-like genes can be found: *clpT1* is highly similar and in close proximity to the *clpC* gene, and probably arose by local duplication, while the unlinked *clpT2* and *clpT3* have a C-terminal Uma2 endonuclease domain. The putative protein SynWH7803_0749 of *Synechococcus* sp. *WH 7803* is probably not a real ClpT, as it is just upstream of a N-truncated ClpB gene and annotated as a possible frameshifted N-terminal fragment. In conclusion organisms where the ClpPR complex is hetero-oligomeric show a tendency to differentiate ClpT and ClpT-like proteins, probably from the Clp-N domain of pre-existing AAA+ chaperones. But most algae dispense perfectly of ClpT, so that ClpT does not appear to be essential to the function of a hetero-oligomeric ClpP complex.

Structure of Cr-ClpP studied by negative staining electron microscopy

Using negative staining electron microscopy, we have investigated the structure of the Cr-ClpP complex, and compared it with that of *E. coli* ClpP. Figure 5a shows a typical EM field and the averaged images obtained by automatic classification of the individual complexes are shown in Fig. 5b. All these views can be construed as a cylindrical barrel with a central stain-filled cavity, flanked by 4 additional masses (arrows in Fig. 5d). The largest of these masses, at 12 o'clock on the image, appears as a comma anchored to the main body by a connector. Smaller masses are found at 6 and 10 o'clock, and in some of the views at 3 o'clock as well. This asymmetry contrasts with the almost perfect rotational symmetry observed for Ec-ClpP (Fig. 5c). Based on the similarity in dimensions of the Cr-ClpP complex and Ec-ClpP, we conclude that the Cr-ClpP complex is basically a tetradecamer, but that its symmetry is obscured with additional subunits/domains forming peripheral masses protruding at the sides. It is noteworthy that no class averages can be interpreted as side-views of a cylindrical barrel suggesting that the complex always adheres via one of its apical surfaces.

Partial trypsinolysis of Cr-ClpP

Probably because of its compact organization, Ec-ClpP is structurally compact and is relatively resistant to treatment by proteases (Li et al. 2010). The more extended structure of Cr-ClpP suggested that proteases might be able to cleave some of the peripheral masses decorating the complex. Trypsin-treatment of the intact complex led to a partial degradation of $ClpP1_H$ and $ClpP1_C$ (and to a lesser extent $ClpP1_{C'}$, based on immunoblotting) and the appearance of immunoreactive bands of smaller MW (Fig. 6). The other subunits, in particular $ClpP1_N$ and the heavily strained ClpR3, ClpR4 and ClpR2, did not appear to be affected. Based on its size, the 30 kDa ClpP1 fragment must be generated by cleavage within IS1. Trypsin-treatment did not lead to the dissociation of the complex, as images obtained by negative staining electron microscopy showed no major difference with the native complex (data not shown).

Discussion

In this paper, we describe the purification of an active chloroplast ClpP complex from *C. reinhardtii*. Homo-oligomeric ClpP complexes have been characterized from a variety of sources, and a ClpP3-ClpR complex from the Cyanobacterium *Synechococcus* was purified by over-expressing the two-gene operon in *E. coli* (Andersson et al. 2009). Recently the hetero-oligomeric ClpPRT core complex of *A. thaliana* has been purified using a Strep-tagII fused to the C-terminal of either ClpP3 or ClpR4 (Olinares et al. 2011). In our hands, attempts to use a His-tag have been met with little success due to massive contamination by proteins interacting with the Ni–NTA resin. Based on Blast searches with the HHHHHH sequence (not shown), we ascribe this failure to the occurrence of a poly-histidine stretch in hundreds of *Chlamydomonas* proteins. We have also used a construct carrying a TAP-tag (Puig et al. 2001; Rigaut et al. 1999), but in this case the yield of the Cr-ClpP complex was found to be too low (<30 μg for 20L of culture, data not shown). Our best success was obtained with the Strep-tagII method described in this paper, which yields in our hands up to 80 μg ClpP per L of culture. This has proven to be sufficient for MS/MS determination of the protein components and for a basic enzymatic and structural characterization of the complex.

Cr-ClpP proved unable to degrade the fluorogenic substrate Suc-Leu-Tyr-AMC. This was also true of the *Synechococcus* complex (Andersson et al. 2009), which also failed to cleave several other short peptides, so it appears that ClpP/R complexes in general are unable to cleave small fluorogenic peptides. It has been proposed recently that *Staphylococcus* ClpP switches between an active extended form and an inactive compressed form, in a cycle that allows release of the degradation products (Zhang et al. 2011). With no substrate in the binding pocket, equilibrium seemed to be infavor of the compressed state. Our results open the intriguing possibility that chloroplast ClpP complex is isolated in a compressed configuration. We do show here that Cr-ClpP cleaves the model peptide FAPHMALVPV at the same position as Ec-ClpP, but only with very low activity. This peptide may have an intrinsic susceptibility to cleavage by ClpP, as it must be removed from Ec-ClpP for protein activation (Thompson et al. 1994). Ec-ClpP (E. Joseph and M. R. Maurizi, unpublished) and *Mycobacterium smegmatis* ClpP (Akopian et al. 2012) are allosterically activated by peptide substrate binding. Cr-ClpP might also be auto-activated for cleavage of certain peptides. The unexpected inhibitory effect of ADEP suggests that surface binding sites on Cr-ClpP have evolved unique responses and have been optimized for specific peptide or protein ligands in *C. reinhardtii*.

Studies by Sjogren et al. (2006) and Olinares et al. (2011) indicate that in Arabidopsis the chloroplast ClpP is composed of two heptameric rings, a P-ring comprising ClpP3, ClpP4, ClpP5 and ClpP6, and a P1/R-ring comprising ClpP1 and all the ClpR subunits. These rings associate in a ~350 kDa "core" complex into which ClpT1 and ClpT2 are also incorporated. Based on results obtained with a Borate-containing native gel system, distinct ClpP and ClpP1-R rings have been proposed to co-exist with a fully assembled core in the chloroplast stroma of Arabidopsis (Sjogren et al. 2006). Using yet a different gel system, the van Wijk laboratory found consistently that the stroma contains a single 350 kDa complex (Peltier et al. 2004). Their affinity-purified complex contained a mixture of the 350 kDa core with two co-migrating 200 kDa complexes corresponding to dissociated ClpP and ClpP1/R rings (Olinares et al. 2011). This was true regardless of whether ClpP3 or ClpR4 carried the tag, implying that dissociation had occurred after affinity-purification of a full-size core complex.

Our experiments with Cr-ClpP shed some light on this issue. CN-PAGE analysis of whole cell extracts and of the purified preparation shows that Cr-ClpP migrates essentially as a 540 kDa core, associating all ClpP, R and T subunits (Fig. 2; Table 1). Dissociation could be observed during purification, especially in the absence of glycerol, but in our final protocol the complex purifies principally as a complete core. A faster-migrating band around 350–400 kDa was also found and its analysis by MS/MS shows that it comprises only the ClpP1/R-ring subunits and ClpT3/T4. Still, it was a minor component, far less abundant than the dissociated rings in the Arabidopsis study (Sjogren et al. 2006). We propose that it results mostly

from dissociation of the core during preparation or electrophoresis. However, because a faint band of similar mobility was also observed in our crude extracts, we cannot exclude that a small fraction of ClpP1/R ring preexists in the chloroplast stroma of *Chlamydomonas*.

From a biochemical point of view, the most salient peculiarity of Cr-ClpP is the presence of multiple forms of the ClpP1 subunit, generated by optional cleavage events. We proposed before that several copies of ClpP1 are found in the complex (Majeran et al. 2005) and later, based on the equal staining intensity of the $ClpP1_C$ and $ClpP1_{C'}$ bands, that three was the most likely number (Derrien et al. 2009). A ClpP1 stoichiometry of three is also in accordance with that observed for ClpP1 by Olinares et al. (2011) in Arabidopsis, and for ClpP3 by Andersson et al. (2009) in *Synechococcus*. Concerning the ClpP-ring, we note that the subunit presenting the lowest coverage was ClpR6, the ortholog of Arabidopsis ClpP6 which also appears to be the least abundant: 1 subunit per ring (Olinares et al. 2011). If Cr-ClpP4 is orthologous to both ClpP3 (with 1 copy/ring) and ClpP4 (2 copies/ring) of plants (Majeran et al. 2005), it is predicted by homology to be present in three copies, and so is Cr-ClpP5.

Comparison of the predicted and observed MW of the various proteins (Table 1) reveals that ClpP1 is probably not the sole ClpP subunit that undergoes processing. ClpR1 was found at a much lower apparent MW than that predicted for its precursor (24 vs. 46 kDa). Determination of the mature N-terminal sequence by Edman degradation showed that 164 AA are removed from the N-terminus. This comprises not only the predicted chloroplast transit peptide (cTP, around 37 AA), but also an N-terminal domain that is conserved in all Chlorophytes (see supplemental Fig 3). In Streptophytes, an N-terminal domain is also found in ClpR1, even though it does not appear to be related to that in Chlorophytes. The observed MW of Arabidopsis ClpR1 (around 28 kDa, Peltier et al. 2001) is also smaller than that computed when the 41 AA cTP predicted by TargetP are removed, and there are no peptides found upstream of position 168 (Olinares et al. 2011), suggesting that also in plants, ClpR1 might be processed beyond its cTP. The MW observed for the mature *Chlamydomonas* ClpR3 (34 kDa) also is much smaller than that calculated for the precursor (47 kDa). But here, the mature protein is almost entirely covered by peptides (suppl Fig 3), which suggests that the difference is due to abnormal migration of ClpR3 in gels. Processing of N-terminal peptides is a critical aspect of assembly of proteasome subunits in proper order and orientation (Chen and Hochstrasser 1996) and it will be intriguing to discover whether cleavage of propeptides is also a mechanism by which the different subunits of Cr-ClpP are assembled into the mature complex.

In every organism where its structure has been studied (except Mycobacteria), the ClpP peptidase shows a barrel-like structure composed of 14 identical subunits (Yu and Houry 2007). The *Chlamydomonas* complex, with 11 different subunits contributed by 8 different genes, does not show this kind of simple sevenfold rotational symmetry when subjected to negative staining and electron microscopy. After image averaging, a certain degree of rotational symmetry emerged, in line with the notion that the overall organization is similar to that of other known ClpP complexes, but it was obscured by the presence of additional peripheral masses. At this stage, we can only speculate as to the origin of these masses. The large comma-shaped mass seen at 12 o'clock on Fig. 5 is the only one that is big enough to represent IS1 (39 kDa). In this view, the presence of one and only one comma-shaped structure per complex suggests that each contains a single unprocessed $ClpP1_H$, along with at least one, or probably two processed copies. The comma-shaped mass is clearly deported towards the outside of the complex, suggesting that the IS1 domain is connected to its attachment site on the apical surface by a long linker. In this position, IS1 does not really "crowd" the upper surface of the barrel, as hypothesized before (Derrien et al. 2009; Majeran et al. 2005).

Smaller peripheral masses were also found at 10, 6 and 3 o'clock. Some may correspond to the remnants of IS1 in

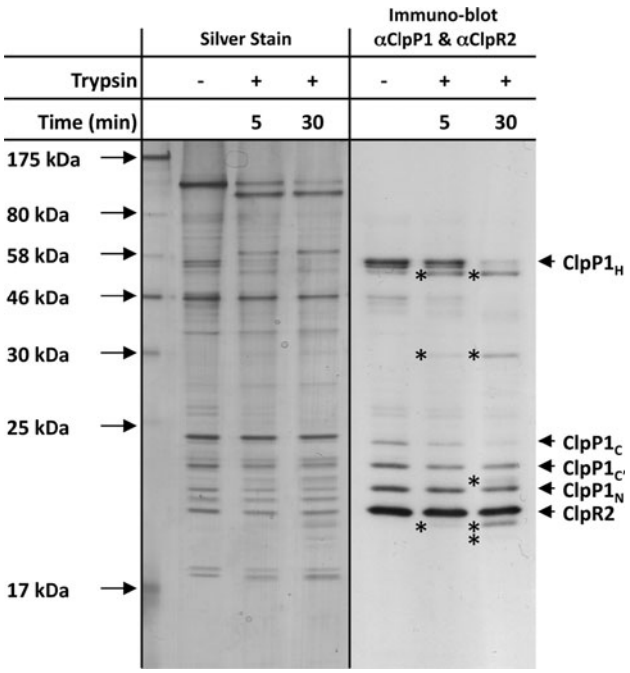

Fig. 6 ClpP1 subunits are more sensitive to trypsin treatment than the other subunits. After trypsin treatment, samples were loaded on SDS-PAGE and either stained with silver nitrate or blotted and probed with ClpP1 and ClpR2 antibodies. ClpP1 tryptic products are marked with an *asterisk*

the processed ClpP1 subunits. According to our partial trypsinolysis experiments (Fig. 6), $ClpP1_H$ is the most sensitive of all the ClpP subunits, presumably because IS1 protrudes at the surface of the complex. But $ClpP1_C$ and $ClpP1_{C'}$ are also sensitive, suggesting that the remnants of IS1 are also exposed on the surface. Some of the peripheral masses would also correspond to additional subunits/domains not found in Ec-ClpP. The C-terminal extensions found in ClpP4 and ClpR3 should be considered, as well as the ClpT3 and ClpT4 subunits.

The plant ClpT subunits have been presumed to serve in substrate recognition and/or regulation of protease activity, based on their containing Clp-N domains (Peltier et al. 2004), or in assembly (Sjogren and Clarke 2011). Interestingly, ClpT genes have appeared independently in plants, in Chlorophycean algae, and in two groups of Cyanobacteria. In addition to any structural role served by ClpT subunits, the fact that they share a Clp-N domain present in Hsp100 chaperones does suggest a role in binding of substrates and/or adaptor proteins. The Clp-N domain of ClpA participates, together with the linker domain, in substrate recognition (Xia et al. 2004). It also plays a crucial role in binding the ClpS adaptor protein, and the residues that are responsible for this interaction are known from co-crystallization studies (Zeth et al. 2002). ClpS interacts with substrates bearing an N-end degron, and reduces the affinity of ClpA for unfolded polypeptides, which makes it a modulator of ClpAP specificity in vivo (Kirstein et al. 2009). ClpS is not present in *Bacillus subtilis*, but substrate binding by ClpC appears to necessitate interaction with various adaptor proteins, of which MecA is the best studied (Schlothauer et al. 2003). Interaction of the Clp-N domain of ClpC with MecA has been mapped to the flexible loop that connects the two halves of the domains (Kojetin et al. 2009). In view of the similarity between the ClpC proteins of chloroplast and *Bacillus*, it is tempting to speculate that Clp-N-domains in the chloroplast ClpC contribute to the binding of adaptor proteins, rather than directly of substrates. MecA has no homologue in the chloroplast, but homologues exist for other adaptors such as ClpS and NblA, an adaptor essential for phycobilisomes degradation in Cyanobacteria (Collier and Grossman 1994; Karradt et al. 2008). Tethering additional Clp-N domains to the ClpPR complex, in the form of permanently residing ClpT subunits, might then increase the diversity of adaptors that can be bound, hence extending the range of possible substrates as well. Further biochemical and genetic studies will be needed to define the role of these subunits in ClpCP proteolysis.

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