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RESEARCH ARTICLE

Dynamics of post-translational modifications and protein stability in the stroma of *Chlamydomonas reinhardtii* chloroplasts

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The proteome of any system is a dynamic entity dependent on the intracellular concentration of the entire set of expressed proteins. In turn, this whole protein concentration will be reliant on the stability/turnover of each protein as dictated by their relative rates of synthesis and degradation. In this study, we have investigated the dynamics of the stromal proteome in the model organism *Chlamydomonas reinhardtii* by characterizing the half-life of the whole set of proteins. 2-DE stromal proteins profiling was set up and coupled with MS analyses. These identifications featuring an average of 26% sequence coverage and eight non-redundant peptides per protein have been obtained for 600 independent samples related to 253 distinct spots. An interactive map of the global stromal proteome, of 274 distinct protein variants is now available on-line at http://www.isv.cnrs-gif.fr/gel2dv2/. *N*-α-terminal-Acetylation (NTA) was noticed to be the most frequently detectable post-translational modification, and new experimental data related to the chloroplastic transit peptide cleavage site was obtained. Using this data set supplemented with series of pulse-chase experiments, elements directing the relationship between half-life and N-termini were analyzed. Positive correlation between NTA and protein half-life suggests that NTA could contribute to protein stabilization in the stroma.

Keywords:
N-α-terminal-acetylation / N-terminal methionine excision / Plant proteomics / Proteolysis / Turnover

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Abbreviations: cTP, chloroplast transit peptide; **FA,** formic acidGlu-tRNA^{Gln} amidotransferase; **Nat,** *N*-acetyl transferase; **NME,** N-terminal methionine excision; **NTA,** N-α-terminal-acetylation; **TMA,** Tris-minimal medium+Arg

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1 Introduction

Proteomes are inherently dynamic in the sense that they are subjected to continuous changes, resulting from regulation of gene expression at all levels, from transcriptional to post-translational. The level at which one chooses to examine a proteome – from the whole organism down to purified subcompartments or complexes – dictates the complexity of the object under study, and hence the resolution power of the methods that will be employed. Protein abundance within a proteome displays a high dynamic range of 4–6 orders of magnitude. In addition to large differences in abundance, proteins also display differences in their stability: some have half-lives in the order of minutes whereas others are much more stable, having half-lives of hours and even days. This property has implications as to the function of a protein. Enzymes that are expressed constitutively are expected to be stable and functional over a long period of time. However, even inherently stable proteins can turnover rapidly when they are damaged due to the exposure to unfavourable environmental conditions. In contrast, proteins that are inherently short-lived are more suitable to act as timing proteins. Once the transcription and translation of the corresponding gene stops, they rapidly disappear. Thus, such proteins are ideal candidates as molecular switches with the potential to regulate all sorts of processes.

Many proteins within a proteome are subjected to a great variety of post-translational modifications (PTMs) during their lifetime and several hundred different types of PTMs have been described so far [1]. If one considers only the N-terminal modifications, they include (i) proteolytic events involving the cleavage of short or long sequences, such as N-terminal methionine excision (NME), leader peptide removal (LPR) and propeptide cleavage (PPC), (ii) N-blocking of the intact or modified proteins, by processes such as *N*-α-terminal-acetylation (NTA) and N-myristoylation (N-Myr), N-methylation or cyclization of the N-terminal Glu or Gln into pyroglutamate, (iii) ligation events extending slightly or considerably the length of the polypeptide chain [2]. These modifications are often crucial as their occurrence may reflect or affect the status, fate and function of the protein. Moreover, several recent studies have pointed out to the participation of N-terminal cotranslational modifications and PTMs, such as NME, NTA and phosphorylation, in regulating protein turnover and as a consequence the dynamics of various proteomes (for a review, see Table 1 in [2]). Hence, PTMs are inherent contributors to the dynamics of proteomes. In this regard, recent large-scale proteomic analyses have pointed out that NTA – which is rare in Prokaryotes – is a major modification occurring in the cytosol of Eukaryotes [3–6].

In the past years, research on the unicellular alga *Chlamydomonas reinhardtii* has come into a new era thanks to the sequencing of its nuclear genome [7]. Particularly, proteomics has became a powerful tool and applied to *Chlamydomonas* to analyze proteins of specific subcellular compartments such as chloroplast thylakoids, mitochondria and flagella, or specific classes of proteins such as the light-harvesting complex proteins, chloroplast thioredoxins, ribosomal proteins or in response to several stimuli (for reviews, see [8, 9]). To date, a 2-DE characterization including the analysis of the *Chlamydomonas* stromal proteome turnover has not yet been reported.

To initiate a comprehensive characterization of protein stability in the chloroplast stroma, their PTMs and the possible link between the two, a proteomic approach was developed. This included 2-DE for separation of the soluble stromal proteins, their identification and the characterization of the PTMs by MS coupled to bioinformatics treatment of the data. We report here that the chloroplast Transit Peptides (cTPs) of the vast majority of the proteins of the stromal proteome are processed, but these sequences are frequently shorter than those found in other organisms containing primary plastids such as in plants [10–12]. We also identify several non-canonical chloroplast proteins that do not undergo removal of their cTPs, but are subjected to other N-terminal modifications such as NME and NTA. Analysis of 80 N-terminal identified semi-tryptic peptides highlights that NTA is the most significant N-terminal protein modification detected out of the searched ones with 30% of stromal proteins concerned. Our data also reveal specific *Chlamydomonas* features of the cTP cleavage and NTA of stromal proteins. The combination of pulse-chase experiments and the above-described 2-DE stromal protein separation has been used to profile the intracellular stability of this *Chlamydomonas* subproteome. Our data highlight that globally the stromal proteome of *Chlamydomonas* cells is mainly governed by the most abundant proteins. Those proteins correspond to stable proteins with a half-life greater than 60 min under standard physiological conditions. A profile has been built that allows exploration of the dynamic of the low abundance stromal proteome in *Chlamydomonas* and investigation of the unstable and highly unstable proteins (half-life less than 60 min). Among them, 66 identifications have been retrieved which allowed determining the first set of *Chlamydomonas* stromal proteins distinguished by a very rapid turnover (i.e. $<$ 30 min). Interestingly, the majority of identified short-lived proteins were proteins whose abundance is known to rapidly and significantly change in response to various signals such as during anaerobic sulfur-depleted H_2 photoproduction [13]. Finally, among all identified proteins with a half-life shorter than 1 h, the NTA was underrepresented.

2 Materials and methods

2.1 Materials

Unless otherwise stated, all chemicals were purchased from Sigma (L'Isle d'Abeau Chesnes, France).

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2.2 Protein extraction and sample preparation

C. reinhardtii (cell wall-less strain XS1 [14]) was grown in TAP liquid medium under constant illumination ($40\,\mu E\,m^{-2}\,s^{-1}$). Intact chloroplasts were prepared as previously described [15] by incubating cells with 0.25% w/v saponin in a lysis buffer containing 50 mM HEPES-KOH, pH 7.5, 5 mM $MgCl_2$ and 0.3 M sorbitol, followed by centrifugation on a two-step Percoll gradient (40 and 80%). Intact chloroplasts were harvested from the interphase (purity was checked under the microscope) and washed twice in the above buffer. They were then subjected to osmotic shock by resuspension in 10 mM HEPES-KOH, pH 7.5, and incubation on ice for 30 min. Chloroplast membranes were precipitated by centrifugation at $100\,000 \times g$ for 30 min, and the stromal extract remaining in the supernatant was frozen in liquid nitrogen. This procedure is schematically summarized in Fig. 1A. To obtain radioactively labelled proteins, cells (250 mL) were grown to mid-exponential phase in TAP+Arg (10 μg/mL) to 2×10^6 cells/mL. Cells were washed in Tris-minimal medium+Arg (TMA) by centrifugation at $4000 \times g$ for 4 min at 20°C, and resuspended at 5×10^8 cells/mL. After shaking for 10 min under $20\,\mu E\,m^{-2}\,s^{-1}$ illumination, ^{14}C-acetate was added (5 μCi/mL) and incubation was continued for 10 min, at which time chloramphenicol (100 μg/mL) and cycloheximide (12.5 μg/mL) were added to stop protein translation. Cells were then diluted in 1 volume of TMA, harvested by centrifugation at room temperature and resuspended in 1 volume of TMA. One quarter of the preparation (T_0 pulse) was resuspended in ice-cold TMA and kept on ice until the chase samples were ready. The remaining three quarters were subjected to radioactivity chase by resuming incubation during 10, 20 or 30 min (T_{10}, T_{20} and T_{30} chase), at which time samples were cooled to 0°C by dilution in ice-cold TMA. At the end of the chase, the four samples were harvested at $5000 \times g$ for 5 min at 4°C. Cells were resuspended in ice-cold lysis buffer, supplemented with 1 volume of 0.5% saponin in lysis buffer and chloroplast stromal proteins were prepared as described above.

2.3 One- and two-dimensional gel electrophoresis (2–DE) of *Chlamydomonas* stroma-enriched fraction

Protein concentration was determined using both the Bio-Rad protein assay kit (BioRad, Marnes-la-Coquette, France) and the 2-D Quant kit (GE Healthcare Life Sciences). For the 1-D gel, the different *C. reinhartii* sample fractions were directly resuspended in 1 volume of Laemmli loading buffer and analyzed on 12–18% acrylamide gels containing SDS and urea [16]. The gels were stained with Coomassie Brilliant Blue (CBB). For 2-D gel of the *Chlamydomonas* stroma-enriched, fraction 5 was diluted directly or after precipitation in trichloroacetic acid (TCA)/acetone [17] in buffer A (final concentration of buffer A: 7 M urea, 2 M thiourea, 0.68% v/v buffer containing 0.27% Biolyte ampholyte, pH 3–10 (Bio-Rad), 0.32% v/v IPG buffer containing 0.128% ampholine, pH 4–7 (GE Healthcare Life Sciences), 0.2% Triton X-100, 2% CHAPS, 20 mM DTT). Several steps of washing of fraction 5 have been added previously to precipitation and solubilization phase to improve the stromal protein focalization. The 18-cm pH 4–7 strips (GE Healthcare Life Sciences) were rehydrated for 24 h at 20°C. IEF was carried out on the Bio-Rad IEF cells at 20°C using a running program previously set up for other plant samples [17]. An adapted focalization profile with progressive increase of the voltage was needed to be set up specifically for the *Chlamydomonas* stroma-enriched fraction: 1 h at 100 V, 3 h at

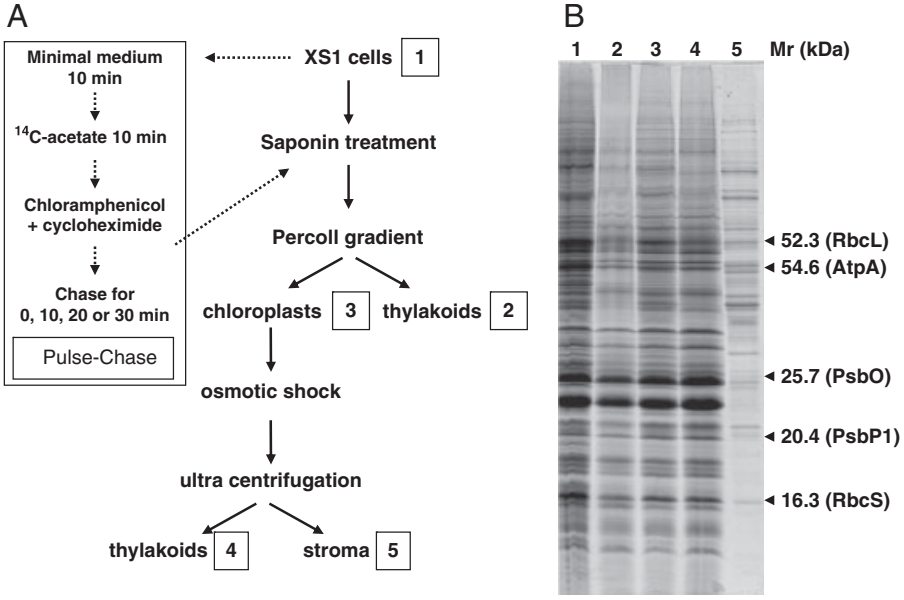

Figure 1. Stroma-enriched fractions from *C. reinhardtii*. (A) Flow diagram for preparation of stroma-enriched fractions including the setup of the pulse labelling experiment on the left. Details of the procedure are described in the text. (B) SDS-PAGE analysis of proteins of different fractions from (A). Samples obtained during preparation of the stroma-enriched fraction were loaded on 12–18% SDS-urea-gel and proteins stained with Coomassie Brilliant Blue R-250. Positions of several chloroplastic proteins are indicated.

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200 V, 4 h at 400 V, 6 h at 1000 V, 10 h increasing between 2000 and 5000 V at 3000 V, 2 h at 5000 V, 2 h at 10 000 V and 2 h at 500 V and a current limited to 50 μA/strip all over the focalization program. The individual strips were washed with MilliQ water (Millipore) and stored at −80°C. Before analysis in the second dimension, strips were equilibrated by incubation for 15 min at 20°C in 5 mL equilibration buffer (6 M urea, 30% w/v glycerol, 2% w/v SDS, 50 mM Tris-HCl, pH 6.8) supplemented with 10 mg/mL DTT and then for 15 min at 20°C in 5 mL equilibration buffer supplemented with 25 mg/mL iodoacetamide. The Ettan DALT System (GE–Healthcare Life Sciences) and 1-mm-thick SDS-denaturing polyacrylamide gels (11%) were used for the second dimension. Gels were run at 50 V for 1 h and 100 V for 16 h at 10°C using a cathodic solution (384 mM glycine, 25 mM Tris, 0.2% w/v SDS) and an anodic solution (192 mM glycine, 25 mM Tris, 0.15% w/v SDS). Gels were stained with silver nitrate (GE Healthcare Life Sciences) or with colloidal CBB [18] and spots were excised for mass spectrometry (MS). Each analysis reported is the result of 3–6 replicates. The gels were scanned with a GS800 imaging densitometer (Bio–Rad) and analyzed with PD–Quest 7.1 software (Bio–Rad as previously reported in [17]) or Progenesis sameSpots software v 3.3 (Nonlinear Dynamics) according the manufacturer's instructions. In the latter case, single stained gels were aligned two by two against a single reference indicated beforehand, with suitable parameters to correct for positional variation from gel to gel. Artefacts were removed with spot area value >1000 in the pre-filter step. The matching was validated after one by one visual checking and manual modification of warping. Replicates gels were put in their group to perform the automatic analysis. Background subtraction performed automatically by the software and normalized volumes were obtained using total volume spot. The same procedure was used for all stained gels and one-way ANOVA were performed. Spots showing ANOVA p-values <0.05 were selected. Radioactive spots were detected by using typhoon (GE Healthcare) after exposition of the 2-DE gels to a storage phosphor screen for 30 days. As proteins were loaded according to total protein dosage and not to total cpm, the total volume spot of radioactive images was not constant. Thus, each radioactive image was normalized by using the corresponding silver staining gel normalization value.

Initially, matching spots were progressively purified by hand. Selected spots were picked with a sterile 1-mL Eppendorf tip.

2.4 LC-MS and protein identification

In-gel digestion was performed with the Progest system (Genomic Solution), according to a standard trypsin protocol or manually as previously described [19]. Rapidly, after gel pieces were washed a few times with successive baths of ACN and 25 mM of ammonium bicarbonate solution, the digestion was carried out for 2–6 h at 37°C. The produced peptides were extracted with increasing amount of ACN acidified with TFA (Progest based) or formic acid (FA) for the manual protocol based. Peptide extracts were dried in a vacuum centrifuge and re-suspended in 20 μL of 0.05% TFA, 0.05% FA and 2% ACN (alternatively in 0.1% FA for manual procedure).

HPLC was performed on an Ultimate LC system combined with a Famos autosampler and a Switchos II microcolumn switch system (Dionex) or a complete Ultimate 4000 system including pump, autosampler and precolumn/column modules. The sample (4 μL) was loaded on a precolumn cartridge (stationary phase: C18 PepMap 100, 5 μm; column: 300 μm id, 5 mm; Dionex) and desalted for 2.5–5 min depending of the system used, respectively. The precolumn was then connected to the analytical PepMap C18 column (stationary phase: C18 PepMap 100, 3 μm; column: 75 μm id, 150 mm; Dionex). Two HPLC profiles were used depending of the instrumentation. With the Ultimate/Famos/Switchos system, peptides were separated using a linear gradient from 5 to 30% B (Buffer A: 0.1% FA, 3% ACN; Buffer B: 0.1% FA, 95% ACN) in 25 min at 200 nL/min. A complete cycle took 45 min, including the regeneration and the equilibration step. With the Ultimate 4000, peptides were separated using a linear gradient from 5–50% B' (in 20 min; Buffer A': 5% ACN, 0.1% FA; Buffer B': 80% ACN, 0.1% FA) at a flow rate of 300 nL/min for a total of 40 min for the entire cycle.

Eluted peptides for the Ultimat/Famos/Switchos system were analyzed on-line with a LTQ XL ion trap (Thermo Electron), using a nanoelectrospray interface. Ionization (1.5 kV ionization potential) was performed with a liquid junction and a non-coated capillary probe (10 mm id; New Objective). Peptide ions were analyzed using Xcalibur 2.07, with the following data-dependent acquisition steps: (i) full MS scan (mass-to-charge ratio (m/z) 300–2000, centroid mode), (ii) ZoomScan on a selected precursor (scan at high resolution in profile mode over an m/z window of 4), and (iii) MS/MS (qz = 0.25, activation time = 30 ms, and collision energy = 35%; centroid mode). Steps (ii) and (iii) were repeated for the two major ions detected in step (i). Dynamic exclusion was set to 60 s.

A database search was performed with XTandem 2010.01.01.4 (http://www.thegpm.org/TANDEM/). Enzymatic cleavage was defined as a trypsin digestion with one possible misscleavage. Cys carboxyamidomethylation and Met oxidation were set to static and possible modifications, respectively. Precursor mass and fragment mass tolerance were +2.5/−1 and 0.5, respectively. A refinement search was added with similar parameters except that semi-trypsic peptide and possible NTA were searched. The *Chlamydomonas* protein database was composed of the Augustus DB Ver. 9 (http://augustus.gobics.de/predictions/chlamydomonas/), plus the chloroplast coded and mitochondrial coded proteins (total of 17 161 entries). Only peptides with an E value smaller than 0.1 were reported. Identified proteins

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were filtered and grouped using X!Tandem parser (http://pappso.inra.fr/bioinformatique.html) according to (i) a minimum of two different peptides was required with an *E* value smaller than 0.05 and (ii) 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 confirmed 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 subgroups.

The Ultimate 4000 HPLC system was coupled to a Q-Trap 4000 instrument (AB Sciex, Concord, ON, Canada) piloted by the Analyst software for data dependant acquisition in the positive ion mode. A basic survey scan from 400 to 1200 Da was followed by an enhance resolution scan to determine the precursor ion charge state. The three highest peaks with a charge state of 2^+ to 4^+ were selected for tandem MS using a predetermined rolling collision energy. Curtain gas (value set to 10) and collision gas (set to high) were nitrogen (Peak N2 generator, Glasgow, UK) and the heated interface was set at 180°C with a declustering potential of 50 eV. Acquired tandem mass spectra were exported from Analyst using the script Mascot.dll (Ver 1.6b23 Matrix Science, UK). In few cases, a Proteome Analyser 4800 (Applied Biosystems) was used to analyze the protein digests as previously described [20]. One microliter of the digested samples was loaded on the instrument target. The same volume of matrix (10 mg/mL CHCA in 50% ACN, 0.1% TFA) was added to the digest on the target. Samples were dried as quickly as possible under vacuum. MS and MS/MS analyzes were performed on the same sample deposit. During MS/MS analysis, air was used as the collision gas. Spectra were obtained by accumulation of 1000 consecutive laser shots were summed for the MS survey spectrum and 2000 consecutive laser shots that were used for the acquisition of the ten most intense peaks of the survey scan. Samples were externally calibrated using standard calibrant mixture. Data were exported using Protein Pilot™ and submitted to on site searching tool (Mascot).

Data processing was done using Mascot tool (Ver. 2.2, Matrix Science, UK) including the carboxymethylation of the Cys as a fix modification and NTA, Met oxidation and pyroglutamination of the asparagines as variable modifications. The database used was the same as the previously described. The peptide tolerance was limited to a maximum of 0.6 Da and the fragment mass tolerance of ± 0.4 Da was applied and the instrument type was set to ESI-QUAD-TOF for the Q-trap 4000 instrument. For the Proteomics analyser, the respective parameters were 100 ppm, 0.2 Da and MALDI-TOF/TOF. A score cut-off of 20 (determined manually by comparison to a search against Human subset database) was applied to the result output to remove most of the false positive peptide characterization. Manual validation of the results was done for each protein.

3 Results

3.1 Challenging a *Chlamydomonas* stroma-enriched fraction with 2-DE

Large amounts of *Chlamydomonas* chloroplasts were prepared using saponin cell lysis and Percoll gradient centrifugation [15]. The stroma fraction was obtained by osmotic shock in hypotonic buffer and ultracentrifugation (Fig. 1A). Upon 1-D-denaturing gel electrophoresis (Fig. 1B and Supporting Information Fig. S1A), the stromal fraction presented a unique pattern clearly distinct from that of thylakoids. However, when we subjected this fraction to 2-DE separation using IEF and gel electrophoresis using classical methods previously employed for other soluble subproteomes [17], extremely poor separation was obtained (Supporting Information Fig. S1B). This was most probably due to the presence of Percoll traces in the stroma-enriched protein fraction and/or the oligomeric nature of stromal proteins. Thus, we introduced several washing steps of the isolated chloroplasts prior to lysis and subjected the stromal fraction to a modified trichloroacetic acid/acetone precipitation [17] to eliminate Percoll traces (Supporting Information Fig. S1C and D). In addition, a dedicated isoelectrofocusing program was set up to overcome the possible issue related to the oligomeric state of the stroma-enriched proteins. All these steps proved to be critical to perform successful isoelectrofocusing of the entire stromal proteome and its second-dimension electrophoresis (Supporting Information Fig. S2). A total of 1785 ± 150 spots were reproducibly detected on silver stained 2-DE gels with apparent molecular weights (M_r) in the range of 10–150 kDa and isoelectric points (p*I*) ranging from 4 to 7. Overall, the pattern obtained with silver staining was similar to that of CBB (not shown) but the sensitivity was higher.

3.2 Systematic protein profiling by 2-DE

We investigated the diversity and nature of the proteins detected in the 2-DE pattern, by analyzing 253 single spots by LC-MS/MS. The majority of these spots were randomly selected as representative of the diversity of spot intensities, M_r and p*I*, as shown in Supporting Information Fig. S2. Statistics for the whole data set are given in Supporting Information Table S1. Most of the spots were picked twice from different replicates of 2-DE gels and the replicated spots characterized by independent MS analyses. The same entries were systematically identified without ambiguity for the replicates with the only slight differences corresponding to total sequence coverage. Analysis of all these spots yielded a total of 600 identifications, corresponding to 274 unique

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entries. We took great care to ensure that MS identifications (average coverage of 26%, with 8 distinct peptides/proteins) were of high quality and unambiguous. All protein identifications and the correspondence between a given spot number and the assigned protein are detailed in Supporting Information Table S1. High sequence coverage made it possible to assign most proteins to a single gene. The data and an interactive version of the identified proteins on a reference gel can be viewed online at http://www.isv.cnrs-gif.fr/gel2dv2/.

Among the 274 identified *Chlamydomonas* proteins, a few (about 11%) are known or predicted to reside in cellular compartments other than the chloroplast (see Supporting Information Table S2). With these data, we can assess cross-contaminations of our preparation by proteins originating from the mitochondrion (7/274, i.e. 2.6%), the nuclei (18/274, i.e. 6.6%) and the cytosol (5/274, i.e. 1.8%). These contaminants included histone H2B, chaperones (HSP70A/C and BIP), subunit H of the glycine decarboxylase complex and a snoRNP component. These putative contaminants (except for the snoRNP component) correspond to either very abundant proteins or are known as very sticky proteins, which are likely to contaminate the stromal fraction during sample preparation. In the case of the acetyl CoA synthetase ACS3 and the superoxide dismutase MSD1, which have been found in the *Chlamydomonas* mitochondrion proteomics survey and whose *Arabidopsis* orthologs have been found in the plastid or in both plastid and mitochondrion, we are tempted to propose a dual localization. A couple of the chloroplast proteins (PsbO, PsbP and several cyclophilins), previously reported to be only luminal, were also retrieved in the stromal fraction, along with two integral thylakoid membrane proteins (PetC, CP26). Based on the M_r and p*I* values of these two proteins as measured on the 2-DE, we exclude that these correspond to degradation products, but they might represent pools of protein en route to their final intra-chloroplast localization or photo-damaged and excluded from Photosystem II complex.

Of the 64 proteins encoded by the chloroplast genome, only 6 were identified in this study (RbcL, EF-Tu, ClpP1, the ribosomal protein Rps9 and the α and β subunits of the CF_1 ATPase). This is expected since the majority of chloroplast-encoded proteins are integral to the thylakoid membrane and because most ribosomal proteins display a strongly basic p*I* value.

Among the nucleus-encoded proteins, a majority (83%, 224/268) presented functional annotation in the Augustus database. This is much more than for the total predicted proteome (39% of 15 951) highlighting the fact that chloroplast biology is a major subject of study in *Chlamydomonas*. We completed the annotation (fields highlighted in cyan in Supporting Information Table S2), using BLAST searches with special emphasis on *Arabidopsis* where subcellular localization is well described by the SUBA database [22]. Also reported in Supporting Information Table S2 is the TargetP results for the *Chlamydomonas* proteins. This program has been trained on higher plant proteins (locating proteins in the cell using ChloroP, SignalP and related tools [23]), and our results show that it clearly over-predicts mitochondrial over plastid localization. Based on their known or proposed molecular function, we generated a prediction of subcellular localization for the 274 identified proteins. Represented in this set were all the classical functions of the plastid: photosynthesis and starch metabolism, amino acid, lipid, pigment and cofactor biosynthesis, protein translation, folding and degradation, nitrogen and sulfur assimilation, etc. Redox control and response to oxidative stress were also represented, along with carbon concentration mechanisms. Four genes involved in the expression of specific chloroplast genes were identified: HCF136, RB38, TAB2 and the protein disulfide isomerase-like RB60, which has been shown to be dually targeted to the plastid and the endoplasmic reticulum [24]. Our preparations also contained cNAP, a nucleosome assembly protein-like that has been found associated with group II introns and could participate in trans-splicing in the chloroplast [25]. We also found 18 proteins previously suspected to be located in either the chloroplast or the mitochondrion (marked as ''organelle'' in Supporting Information Table S2) and for which the *Arabidopsis* homologue was usually annotated as plastidic.

For the majority of the proteins in our set, however, this new map of the stromal proteome of *Chlamydomonas* provides important clues to their localization hence their function. Of particular interest are eight proteins that are part of the ''GreenCut'' [7], a set of proteins of unknown function but whose conservation in photosynthetic organisms suggests an important role in chloroplast biology. We also found LPB1, a protein important for acclimation to phosphorus and sulfur deprivation [26], a putative PII protein uridylyl-transferase that might participate in the control of nitrogen metabolism [27], and two polyketide cyclase/dehydrases, suggesting that type II polyketide synthesis occurs in the chloroplast. Along with several proteases and aminopeptidases, we noted the presence of enzymes participating in amino acid degradation (isovaleryl-CoA dehydrogenase, methylmalonate semi-aldehyde dehydrogenase and 3-hydroxyisobutyrate dehydrogenase), suggesting that the chloroplast is an active site of amino acid recycling in *Chlamydomonas*. The plant orthologs of the first two enzymes have been found in mitochondria. The carbon skeletons generated by amino acid degradation can be used for gluconeogenesis, and it is interesting to notice that our chloroplast preparations contained a key enzyme of this pathway, phosphoenolpyruvate carboxykinase, which is found in the cytosol in *Arabidopsis* [28] and other organisms.

Along with several aminoacyl-tRNA synthetases, we found a protein with high similarity to the C-subunit of Glu-$tRNA^{Gln}$ amidotransferase (GAT). In the absence of an organellar Gln-tRNA synthetase, $tRNA^{Gln}$ is acylated by a GluRS and the Glu moiety is converted to Gln by GAT [29]. A one-subunit enzyme has been purified from *Chlamydo-*

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monas [30] but its intracellular localization has not been investigated and the genes were not characterized. In addition to the GAT3 gene encoding subunit C, the genome contains a GAT1 gene encoding subunit A, but no homologue of subunit B. The *Arabidopsis* GAT subunits have been proposed to be dually targeted [29], but no trace of either GAT1 or GAT3 have been found in the *Chlamydomonas* mitochondrial proteome. Finally, enzymes involved in PTMs were encountered in the form of an Arg methyltransferase and several kinases and phosphatases.

3.3 New clues on transit peptide processing in *C. reinhardtii*

It should be reminded that previous work on cTPs in plants led to the development of several artificial neuronal network-based programs capable of remarkable accuracy [11, 31]. Unfortunately, the prediction of cTPs in *Chlamydomonas* has remained a bottleneck of all available computational predictors because (i) the sequence conservation in signal peptides is very low, (ii) the existence of a slightly different transit peptide-receptor recognition mechanism in *C. reinhardtii* and (iii) none of the existing bioinformatics prediction tool were trained with *C. reinhardtii* data sets [32]. Our MS data including semi-tryptic peptides search in Mascot and/or X!Tandem, followed with manual verification and M_r variation analysis of the 2-DE, highlight some new clues concerning the CTPs in *Chlamydomonas*.

Comparison of the experimental M_r of the whole set of identified stromal proteins to their predicted values (Supporting Information Table S1) shows only poor correlation between the two values (Supporting Information Fig. S1E). In many cases, we noticed that the identified proteins displayed an experimental M_r lower than the theoretical one. Indeed, we observed that the experimental protein M_r was on average 2.1 kDa smaller than the expected one. This is markedly less than the 6.6 kDa observed in *Arabidopsis thaliana,* suggesting that the length of cTP in *Chlamydomonas* could be shorter. Although M_r is difficult to determine experimentally, a much more detailed description emerges from analysis of the sequences. Indeed, the observed M_r discrepancy was further confirmed by the identification and characterization of 189 unique putative stromal N-termini (Supporting Information Table S3A). To define these possible N-termini, all MS data were analyzed allowing tryptic and semi-tryptic cleavage with optional N-terminal PTMs including NTA. In our study, N-terminal semi-tryptic peptides are peptides, which were cleaved by trypsin at the C-terminal side of R or K but not at the N-end. These peptides therefore result from an endoproteolytic cleavage or correspond to the N-terminal peptides. Due to the potential bias introduced by the tryptic peptides in such approach for the identification of the protein N-terminus, we took into account only those identified at positions below the 60th amino acid. In all cases, we made sure that no other peptide was found upstream in the sequence. Based on these experimental data (Supporting Information Table S3A), the average cTP cleavage site is located on average in alpha of the amino acid position 37 ± 14 ($n = 189$). Similar results were obtained when we considered only the 87 semi-tryptic N-termini (NTA or not) with an average cTP position at 38 ± 13 or the only 25 N-α-terminal-acetylated protein peptides, which could be considered as unambiguous true N-termini due to their PTM at the N-termini (34 ± 7, see Supporting Information Table S3A). In addition, these data are in full agreement with the few previously identified ribosomal N-termini (38 ± 14; [33]), which we used together with our N-termini data set to construct sequence logos of the cTP cleavage site between positions −12 and +9 (Fig. 2 and Supporting Information Table S3B). As previously shown in *Arabidopsis*, using the full set of tryptic and semi-tryptic peptides, we also observed a false enrichment of R and K at position −1 (Fig. 2A). However, this bias was obviously not visible anymore when only semi-tryptic peptides were used (Fig. 2B–D). In these latter cases, the sequence logos (Fig. 2B and C) emphasize positions +1, −4 and −9 for their enrichment in Ala, Val and Arg residues, respectively. These data highlight that there is a strong preference and/or conservation for these amino acids around the cTP cleavage site. This observation was further supported by the more stringent dataset related to the N-α-terminal-acetylated proteins. From the N-α-terminal-acetylated proteins, the protein sequences around their respective experimentally determined N-termini (± 21 amino acids) were aligned in a sequence logo plot (Fig. 2D and Supporting Information Table S3B). This illustrates conservation at the position −1, −2, −3, −7, −8, −9, and −12 ([A/R], [A/V,T], [V], [R], [R], [R,V], [R,A], respectively), as well at position +1 ([A/V]) and +2 showing a clear and unique preference for Alanine at this position (Fig. 2D). Taken together, all these amino acids positions likely represent a combination of a consensus motif for the *Chlamydomonas* cTP that seems to share some similarity with the consensus motif for *Arabidopsis* but also highlights some unique features (see Section 4 and [12]). An enrichment for R was observed when creating the sequence logo (Fig. 2D) as previously shown for *Arabidopsis* when using a large data set [12]. However, our experimental data show that this enrichment is localized at positions −7, −8, −9, i.e. closer to the rice than to the *Arabidopsis* profile [12].

3.4 N-α-terminal protein acetylation as a recurrent modification of the N-termini of stromal proteins

As recently shown for the *Arabidopsis* counterpart [12], we also observed in the *Chlamydomonas* stroma *N*-acetylated peptides with a prevalence of A, V, I, S, T at the position P'1 of the cleavage site (for the definition of the P_n/P'_n cleavage site of polypeptide sequence, see [34]). When the N-terminal

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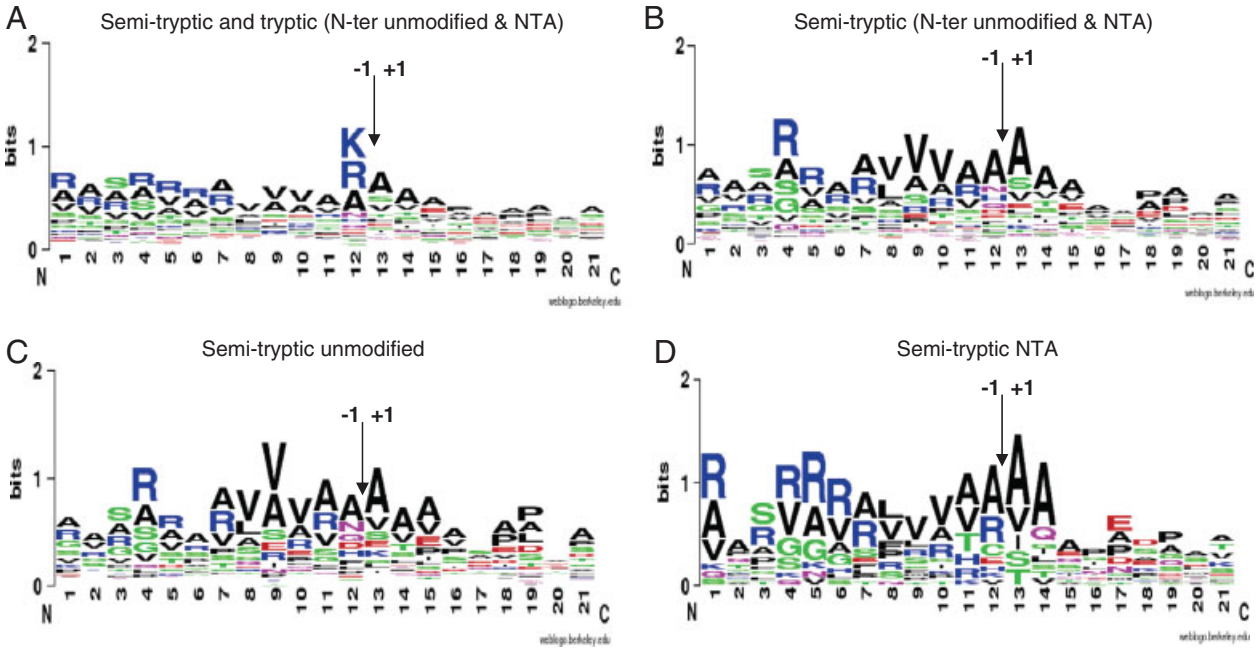

Figure 2. Sequence logos around experimentally determined N-termini. (A) Sequence logo was constructed with the 21 residues surrounding the cTP cleavage position from 194 putative MS-identified N-termini (tryptic and semi-tryptic peptides). (B) Sequence logo was constructed with the 21 residues surrounding the cTP cleavage position from 92 semi-tryptic MS-identified N-termini. (C) Sequence logo was constructed with the 21 residues surrounding the cTP cleavage position from 66 semi-tryptic unmodified MS-identified N-termini. (D) Sequence logo was constructed with the 21 residues surrounding the cTP cleavage position from 26 NTA MS-identified N-termini (semi-tryptic and tryptic). NB: in (A), (B) and (C), five MS-identified N-termini from *C. reinhardhtii* chloroplast ribosome proteins were added to the logo analysis.

residue corresponds to an Ala residue, usually only partial NTA occurred (i.e. the same peptide has been retrieved acetylated and non-acetylated). In contrast, when the first amino acid was Val, Thr or Ser, NTA was complete. Unlike *Arabidopsis,* for which the conservation at position +3 (P'3, see Fig. 6A in [12]) was suggested to be due to the preference for the N-terminal acetylase rather than the stromal processing peptidase (SPP), no similar conserved residue was found at the same position in *Chlamydomonas*. Thus, this position should not be taken into account to define the consensus motif for both *N*-acetyl transferase (Nat) recognition and for the stromal processing peptidase in *Chlamydomonas*.

By comparing the experimental M_r – as measured from 2-DE separations – to its theoretical value (based on database sequence entry), we also observed that a protein set showed no difference. This suggests that these proteins retain their transit peptides, possibly because they have a non-canonical targeting sequence as previously reported in other organisms or for some of the chloroplastic ribosomal proteins of *Chlamydomonas* [33, 35, 36]. Indeed, among the N-termini characterized by MS, we have identified 15 proteins where the N-terminus was observed at position +1 to +7 of the predicted protein (Supporting Information Table S3). A thorough analysis of all retrieved sequences suggested the absence of a cleavable transit peptide for at least five of them. Furthermore, three of the N-termini of this set appear to have arisen from the rules of NME cleavage [37],] i.e. the first Met is excised when the second amino acid has a small side chain such as Ala or Ser, and no cleavage of the first Met is observed when the second amino acid is bulky [3, 37]. These three proteins correspond to the METM *S*-adenosylmethionine synthetase, the AST2 aspartate aminotransferase and the fungal-type translation elongation factor EF3. These three proteins were found to be also N-terminal α-acetylated (Supporting Information Table S3). Although the latter two proteins have been tentatively annotated as cytosolic based on function, we propose that at least a fraction of these proteins reside in the chloroplast stroma where they are imported by an unconventional import process that does not remove an N-terminal extension, followed by NTA. Chloroplast import without cTP cleavage has been already recognized as a quantitatively significant process [35, 36] and in *Chlamydomonas* the chloroplastic ribosomal proteins PRPL2 and PRPL14 apparently lack a cleavable transit peptide [33].

3.5 Characterization of the half-life of stromal proteome of *Chlamydomonas* cells

Despite the growing interest in the dynamic nature of proteomes, there is currently little knowledge of the protein framework responsible for the global half-life of the stromal

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proteome of *Chlamydomonas* cells. This includes the comprehensive array of the stromal proteins with short half-life, a set of strongly interesting proteins, as this may suggest high instability due to oxidative sensibility or regulatory roles. Thus, the identification of short-lived proteins in the stroma of *Chlamydomonas* cells could give information on the half-life of many regulatory proteins, on signals for rapid degradation, which are largely unknown and generally on the stability/instability of this subproteome. Our methodological choice of analyzing this subproteome by 2-DE allowed us to investigate these aspects as a function of the M_r, p*I* and cotranslational modifications and PTMs affecting specifically each identified protein.

In this context, pulse-chase experiments in the presence of ^{14}C-acetate were combined with proteins synthesis inhibitors to determine the global stability/instability of the stromal proteome. We chose a short exposure time (10 min) to the labelled precursor to increase our ability to detect proteins with high turnover rates and to maximize their relative labelling compared to those of the most stable proteins [38]. To initiate the chase, protein synthesis was blocked by specific inhibitors. Time-course analysis for 30 min (T_0, T_{10}, T_{20}, T_{30}) of the 2-DE silver-stained stromal proteome revealed that a minority of the accumulated detected proteins are short half-lived. Indeed, the number of detected spots was gradually reduced after 30 min to about 70–80% (~450 spots) of the total detected proteome at time T_0 (Fig. 3A and B). This is consistent with an average half-life value being more than 1 h when we consider the whole spot population. When checked manually one by one, the number of spots for which differences has been highlighted during time-course experiments drastically decreased by a

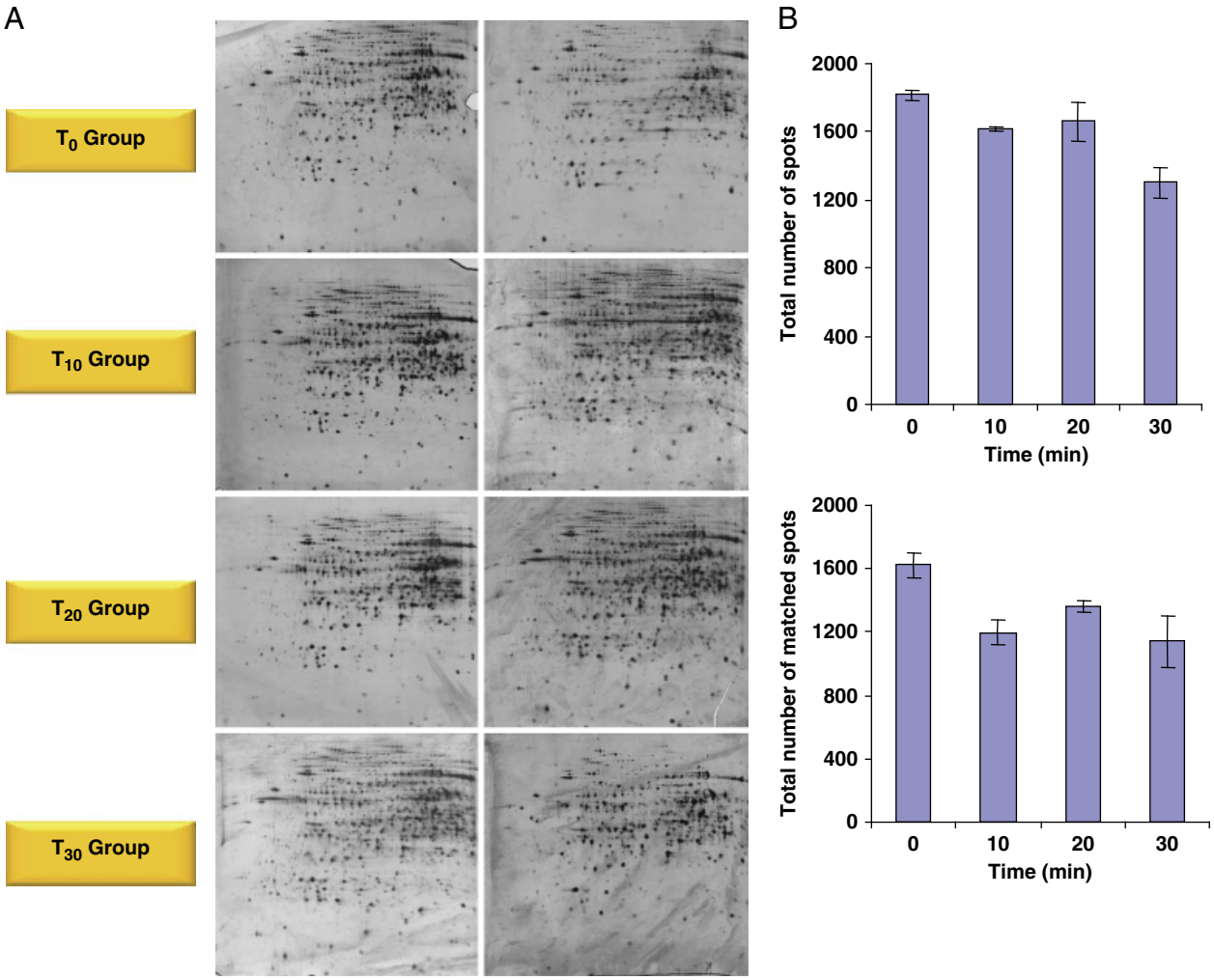

Figure 3. Protein stability profile of the stromal proteome. (A) kinetic analysis (T_0, T_{10}, T_{20}, T_{30}) of the stromal proteome by combination of pulse-chase experiments and 2-DE. Pulse-chase experiments, in the presence of ^{14}C precursor, were combined with proteins synthesis inhibitors. Stromal-enriched fractions corresponding to different times were prepared and subjected to 2-DE as previously described (see Section 2 and Fig. 1). Two replicates are shown for each group. Spots were visualized using silver nitrate staining. (B) The number of spots detected at each time was calculated using Progenesis sameSpots software v 3.3 (Nonlinear Dynamics). Total number of matched spots was calculated by aligning the single stained gels two by two against a single reference.

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factor of 10. The majority of them belonged to a class of low abundance proteins, barely detected with the most sensitive on-gel protein dyes (Supporting Information Fig. S3). Thus, these data suggest that the overall temporal dynamics of the stromal proteome of *Chlamydomonas* cells is mainly governed by the most abundant proteins. Those proteins correspond to stable proteins with a half-life longer than 30 min under standard physiological conditions.

Thanks to the fact that the 10-min ^{14}C pulse set before protein synthesis was halted by specific inhibitors, we could investigate and characterize highly unstable stromal proteins (half-life of less than 30 min). A profile can be built that allows exploration of the dynamic of the low abundant stromal proteome in *Chlamydomonas*. The very short pulse allowed the detection on the 2-DE gel of 452 radioactively labelled spots at T_0, most of which were not detectable by silver staining of the same gel (Supporting Information Fig. S3–S6), indicating that they correspond to low abundance proteins. As expected from a short exposure to a labelled precursor (e.g. proteins having a high turnover rate are highly labelled, whereas stable proteins are less labelled and their turnover rate is not revealed), 84% of the labelled spots were unstable and among them 62% (235) were found highly unstable (Fig. 4). These highly unstable proteins/spots could be dissociated into three subclasses of (i) 32 spots (14% of the unstable protein set) with an average half-life of 8 ± 2 min (Fig. 5), (ii) 109 spots (46% of the unstable protein set) with a half-life between 10 and 20 min and (iii) 93 spots (40% of the unstable proteins) with half-life average of 25 ± 3 min (Fig. 5 and Supporting Information Table S4). The remaining unstable proteins, i.e. 147 spots, displayed a half-life ranging from 30 to 60 min. Finally, no variation of the labelling level was observed within the 30 min of chase experiment for the 71 remaining spots (Supporting Information Fig. S3–S5 and Supporting Information Table S4).

Because of the extremely low abundance at the steady state of the majority of the labelled proteins, particularly those with extremely rapid turnover, it was very difficult to pick such spots and to characterize the corresponding proteins by MS. Nevertheless, among the 452 quantified

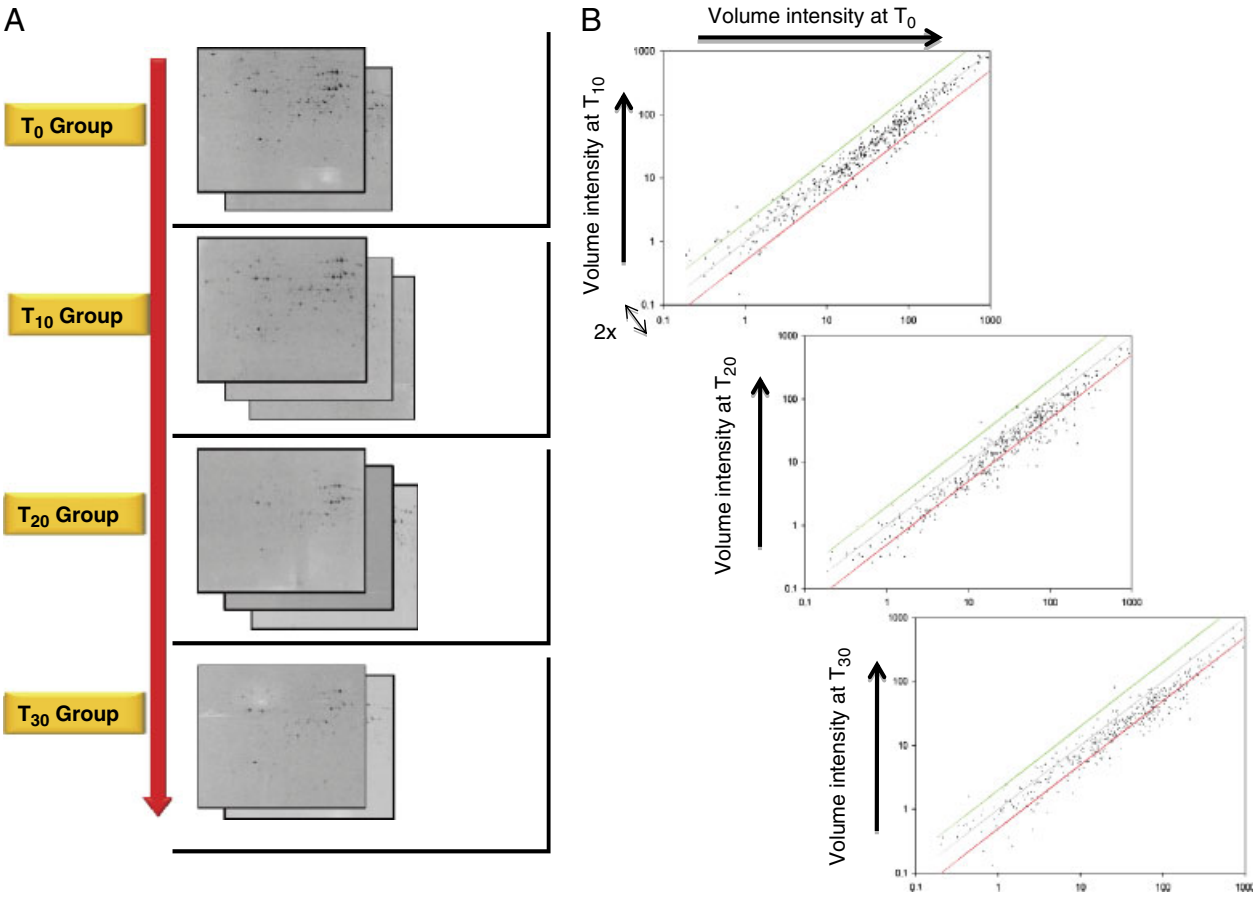

Figure 4. Outline of highly unstable *Chlamydomonas* stromal proteins. (A) Radioactive 2-DE gel images corresponding to the kinetic times of the pulse-chase experiments. Replicate gels were assembled according to their group time. (B) For the Scatter Plot, the group sets of replicate gels in (A) were used to determine the average volume intensity of their protein spots. The average volume intensity of each radioactive spot belonging to T_0 group (*x*-axis) is plotted on a log scale against its average volume intensity in gels belong to other groups (*y*-axis).

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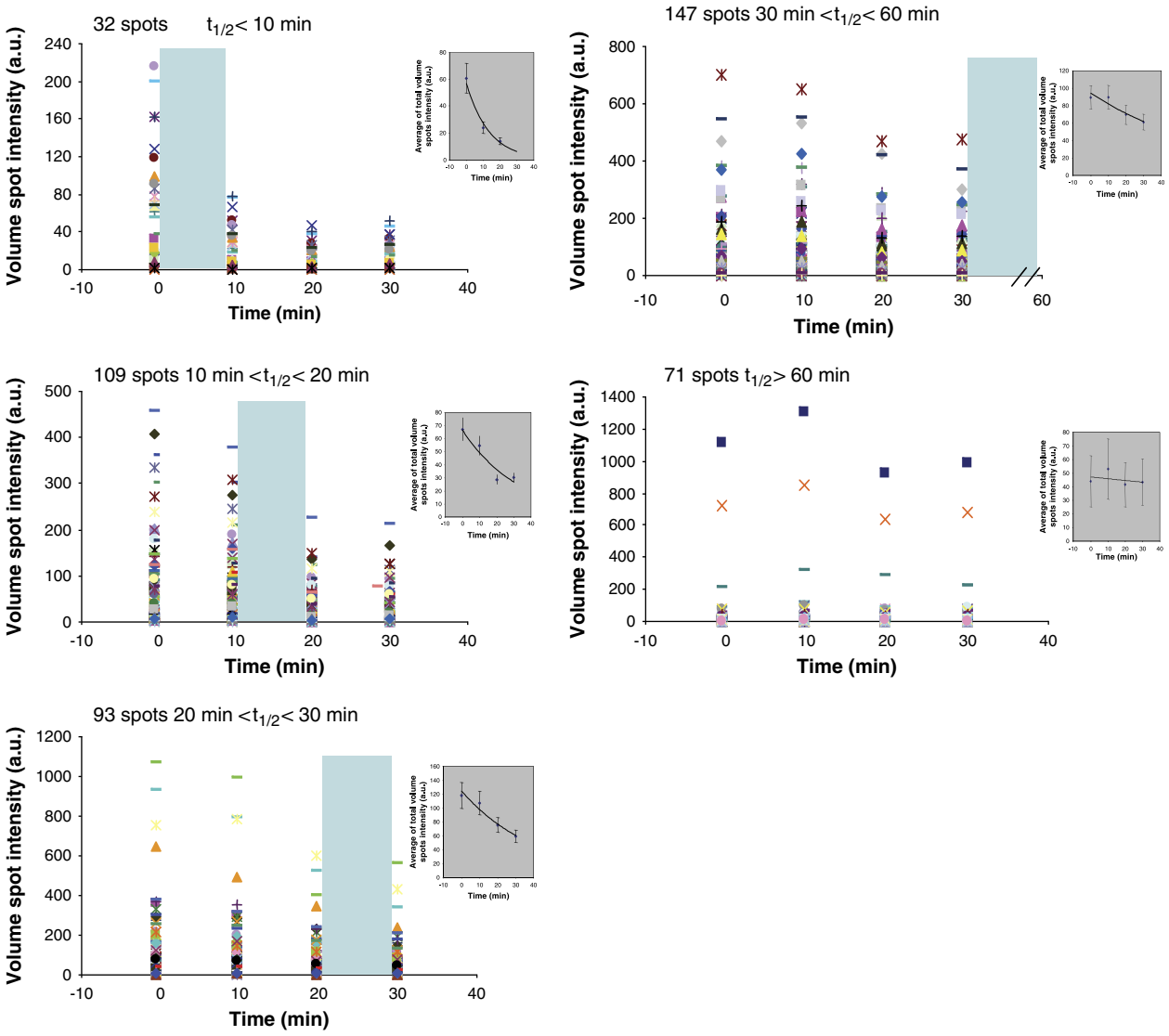

Figure 5. Measurement of turnover rate of stromal proteome of *Chlamydomonas* cells. The average volume intensity of each radioactive spot normalized using the corresponding silver staining gel normalization value were followed over the time of the chase experiments and spots with the same behavior (showing the same turnover) were grouped in the same graphic. To obtain the average turnover rate of mixed spots showing same tendency, the average of total volume spot intensity was plotted on a linear scale and reported in the small graphic. The mean half-lives ($t_{1/2}$) were calculated from the exponential curve generated from the plot.

spots, 107 matching spots both radiolabelled and silver stained spots could be characterized by MS/MS. This set included spots featuring rapid turnover (Supporting Information Fig. S5 and sheet "Correlation" in Supporting Information Table S4).

Among them, 66 spots have been identified (see Supporting Information Tables S1, S2 and S4), which allowed determining the first set of *Chlamydomonas* stromal proteins distinguished by a rapid turnover (i.e. <30 min). Spots corresponding to proteins with the highest turnover (i.e. <10 min) were very difficult to characterize because of their low abundance (see above). However, three proteins of them have been successfully identified and corresponded to the iron superoxide dismutase FSD1, a HSP70-like protein and a protein with unknown function. More proteins can be characterized in the pool of spots displaying a half-life between 10 and 20 min (59 proteins, Supporting Information Table S4). Interestingly, the majority of the identified short-life proteins were proteins whose abundance is known to rapidly and significantly change in response to various signals such as sulfur deprivation and H_2 photoproduction [13]. Focusing further on these proteins, we could identify 24 N-terminal peptides (6 tryptic and 18 semi-tryptic one). All of them were found to be free of NTA, except two, both partially N-α-terminal-acetylated (Au9.Cre02.g085450.t1, a coproporphyrinogen III oxidase, and Au9.Cre07.g339700.t1,

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an iron-sulfur cluster assembly protein). Thirty-four spots were also characterized in the pool of proteins with a turnover between 20 and 30 min (Supporting Information Table S4). Many of the identified proteins of this group coincided with proteins belonging to known megadalton complexes (in bold in Supporting Information Table S4) such as plastid pyruvate dehydrogenase (PDC), acetyl carboxylase (ACCase) and ribosome (use [39] for discussion). Among the identified proteins in this pool, only nine putative N-terminal peptides including six tryptic and three semi-tryptic fragments were retrieved. Interestingly, for the semi-tryptic peptides, two have been again found to be not acetylated (both related to a HSP70C that likely underwent to the removal of its cTP) and the third one (one of the few cases of unprocessed proteins, Au9.Cre06.g250200.t1) was N-α-terminal-acetylated. Concerning the pool of spots with a turnover between 30 and 60 min, we could identify 33 N-terminal peptides (16 tryptic and 17 semi-tryptic). Again, as for the other pools of proteins with short half-life, only three of the N-terminal peptides were retrieved N-terminal acetylated (Supporting Information Table S4). Finally, among the 71 spots for which no variation of the labelling was observed, 15 spots were characterized and 9 N-termini (3 tryptic and 6 semi-tryptic) were identified. Among these, only one semi-tryptic peptide was found to be N-α-terminal-acetylated.

Interestingly, through all identified proteins with the shortest half-life (i.e. less than 1 h), N-α-terminal-acetylated proteins were underrepresented compared to whole pool of identified semi-tryptic peptides. This under-representation (6/38 proteins) needs to be compared with the 26/87 value observed in our random characterization of the most abundant spots retrieved by 2-DE. It appears therefore that there is a positive correlation between the frequency of NTA and stromal protein abundance and an opposite correlation between this modification and protein turnover (i.e. proteins with a high turnover are less N-α-terminal-acetylated than proteins with a low turnover).

4 Discussion

4.1 New information on the *Chlamydomonas* stroma proteome

A cellular or subcellular proteome is the product of a dynamic process combining not only the well-known protein synthesis and degradation processes but also cotranslational modifications and PTMs. Thus, a given ''global proteome'' is an instant picture that provides a static overall description of cellular protein content with no information on its dynamic features (e.g. the half-life of the whole set of proteins recovered in the proteome). Quantitative or semi-quantitative proteomics can be used to compare proteomes in different conditions, but this reflects mainly changes in gene expression, more than the dynamics of the proteins themselves. We attempt here to not only list the major proteins of the chloroplast stroma in *Chlamydomonas* but also to approach their dynamic properties in standard growth condition, in terms of processing, N-terminal modification and stability.

In the last few years, the release of the nuclear genome of the green alga *C. reinhardtii* has boosted up proteomics studies in this important model organism showing both animal and plant features [8, 40]. Comprehensive lists of components of several subcellular compartments (protein complexes and phosphoproteome of the thylakoid membranes, chloroplast ribosome, eyespot, cilium, redox targets and soluble proteins) have been already provided [9, 13, 33, 41–48]. However, no experimental data regarding the chloroplast stromal proteome of *Chlamydomonas* or its stability have been reported until now.

Although 2-DE technology is associated with known drawbacks such as the difficulty of sample handling and low throughput, it remains unique in its ability to separate proteins at high resolution when applied to a subproteome of moderate complexity, as is the stromal proteome. This approach is able to clearly separate protein with various isoforms and/or PTM through the M_r and pI ranges. Accordingly, we have developed a rigorous 2-DE stromal protein profiling, coupled to MS analysis and pulse-chase experiments in order to investigate the intracellular stability of the stromal proteome of *Chlamydomonas*. An interactive map of the global stromal proteome (http://www.isv.cnrs-gif.fr/gel2dv2/) including 274 protein variants is presented here, thus providing a great deal of information. Note that a most recent high-throughput analysis of the whole chloroplast proteome under anaerobic response in *C. reinhardtii* using gel-free MS reported 895 plastid proteins [49]. In this large set, only 114 entries of our data set (42%) were identified, e.g. our study reported 160 new entries. In addition, extrapolation from the higher plant data suggests that close to 2000 of the 15 000 *Chlamydomonas* proteins are chloroplast-localized. This indicates further that different strategies are required to get full insight of a subproteome complexity such as that of the stroma.

4.2 Comparative analysis of the protein content of the stroma in algae and land plants

The stromal proteome of higher plants, maize and *A. thaliana*, has been already investigated through the combination of 2-DE separation and MS analysis in the course of comparing mesophyll and bundle sheath [50] or studying the oligomeric state of proteins [51]. More recently, high-throughput analysis based on LC-MS/MS protein characterization has been performed [12]. These studies provided a wealth of information on the major proteins contained in the stroma of specialized cells of multicellular plants. Our study instead focuses on a unicellular green alga, separate from the former early in evolution of the green lineage. Yet, the stromal protein content of

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Chlamydomonas appears to be very closely related to that of *Arabidopsis* (for cross-correlation, see Supporting Information Table S2). For almost every protein in our set, we identified a corresponding homologue in *Arabidopsis* and for most of them experimental data were available. Noticeably, we have recovered information for 48 proteins (i.e. 17% of our set of identified proteins), which do have an *Arabidopsis* homologue, but for which no data had been described to date. Among these proteins, several display no function predicted in both *Arabidopsis* and *Chlamydomonas* (for example, LCIB, LCI3 and a protein similar to LCI9, whose induction by low CO_2 suggests a role in carbon concentration or fixation); the others correspond to newly identified stromal proteins that could be involved in plastid metabolism, translation, folding, wounding/stress response and proteolysis. In this latter category, we note the presence of an OTU-like cysteine protease family proteins, metalloendopeptidases (including PREP1, a peptidase responsible for the degradation of targeting peptides in mitochondria and chloroplasts), peptidase M1 family proteins as well as a polyketide cyclase/dehydrase, and an unusual protein with a CBM20 carbohydrate-binding module.

4.3 Cotranslational modifications and PTMs in the stroma of *Chlamydomonas*

Our present study provides a wealth of new information dealing with protein modifications occurring on the way to or in the stroma. This includes proteolytic events and NTA, two major N-terminal modifications [2]. The involvement of NME could be also studied thanks to the availability of several N-terminal sequences for which the N-terminus clearly originates from methionine aminopeptidase (METAP) specificity (see Supporting Information Table S3). All these peptides that underwent NME were also N-α acetylated (see bottom of Supporting Information Table S3). While we cannot rule out that these were in part due to contamination by cytosolic proteins adhering to the chloroplast envelope, we have to consider the possibility that these proteins are imported into the chloroplast without cleavage of an N-terminal sequence. This raises the question of where it exactly occurs. Does NME of these proteins take place on the ribosome in the course of cytosolic protein synthesis and before translocation, or post-translationally by plastid NME enzymes in the stroma? This is an important question that expands the recent debates concerning cotranslational events and the ''welcome commitment'' of the ribosome-associated protein biogenesis factors (RPBs), which clearly includes the NME enzymes [52, 53]. More work is needed to determine where imported chloroplastic proteins are processed by the NME enzymes. The question is simpler for NTA of proteins that undergo previous removal of their cTPs. In this case, NTA is likely to involve at least one dedicated enzyme, as proposed earlier [54, 55]. NTA has been found in the cytosol, and also in the chloroplast. This process is most likely controlled by a stromal Nat, which is believed to work post-translationally [54, 55]. We assess that about 30% of stromal proteins undergo this PTM, a percentage similar to that found in other organisms such as yeast or some Archaea [3]. Regarding the specificity of Nats, even if the *N*-Ala was the most frequently N-α-acetylated amino acid, we also retrieved Thr, Val, Ser, Cys, Glu and Asn proteins as N-terminal acetylated residues. This might suggests that in the stroma of *Chlamydomonas* several Nats, corresponding to NatA, NatB and NatC found in other higher eukaryotes or to the three Nats of *Escherichia coli* (RimI, RimJ and RimL; see [56]), could exist. These enzymes could also be responsible for the NTA of chloroplast-encoded proteins such as RbcL, D1 or D2 (respectively on Ser and Thr residues). Among the 38 *N*-acetyl transferases annotated in *Chlamydomonas*, a dozen is predicted to be located in the chloroplast, among which Cre01.g002250 shows highest similarity to RimI. Whatever their number and identity, these stromal Nats do not seem to display any preference for a given residue at position +3. This is unlike the situation in *Arabidopsis* for which a conservation of a Ser residue at this position was noticed [12].

Most of the proteins that we retrieved in the present study originate from nuclear-encoded proteins. In plants and algae, 15% of the open reading frames of the nuclear genome are believed to be targeted to the chloroplast. This corresponds to a total of 1000–4000 proteins (2000 in *Chlamydomonas*) depending on the size of a proteome, which is 2–3 times larger in higher plants than in algae. This number needs to be compared to the only 60–100 open reading frames usually encoded by the plastid genome (64 in *Chlamydomonas*). Whatever their origin, plastid proteins are often highly abundant and represent a major fraction of the total cell protein content. In terms of mass, the impact of their synthesis and of their import is therefore major in plants and algae.

As already noted by others [33, 35, 36], we found several stromal proteins in *Chlamydomonas* that do not display a cleavable transit peptide (see Supporting Information Table S3). While in some cases, we have found that the gene model does not accurately represent the translation start, at least five cases unambiguously show absence of any cleavable cTP. It is not clear whether these cleavable cTP-less proteins are imported by the classical TOC/TIC pathway or by a totally different route, but these proteins clearly deserve further study. When occurring, we show here that the length of cleavable cTPs in *C. reinhardtii* proteins is much shorter (34 ± 7 residues) than that of *Arabidopsis* (59 ± 23 residues). In addition, the consensus around the cleavage site is significantly different (Fig. 3). This could mean that the cTP peptidase specificity is different or reflect the fact that the properties of the cTP and the import machinery are quite divergent between the two organisms [12, 57]. As shown in Supporting Information Table S2, TargetP/ChloroP, the most efficient software to predict cleavage sites

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of plastid proteins in higher plants [23],] appears very inefficient in providing robust prediction of cTP in *C. reinhardtii*, as shown in Supporting Information Table S2. This is unlike the situation in *Arabidopsis* where large-scale analysis of thylakoid membrane proteins has revealed that 89% showed a cTP predictable by TargetP [54, 58]. Nevertheless, our study shows that *Chlamydomonas* transit peptides are closer to those of higher plants – although with a significantly lower Ser/Thr content – than to those from red algae or diatoms, as already suggested (see logo plots available in Table 1 of [10]).

We noticed that unlike the most abundant plant plastid proteins which usually have an Ala as second residue of the full-length transitory protein [3, 59], the situation was different in *Chlamydomonas* for which no real conserved residue has been observed with a mixture of Ala, Val and Thr residues being equally represented at this position. In this respect, as well as in other aspects such as length and amino acid composition, our data also confirm that *Chlamydomonas* cTPs look more similar to plant mitochondrial targeting sequences [57]. We also have noticed that TargetP tends to frequently predict mitochondrial targeting for those sequences. Such cTPs appear not to have arisen nearly at random, as it appears to occur also in apicomplexa such as *Plasmodium falciparum* [60]. Indeed, we observed a large over-representation in Ala, Arg and Ser residues in *Chlamydomonas* cTP. This indicates that dedicated prediction tools are required for properly predicting cTP cleavage in chlorophytes such as *C. reinhardtii*, red algae or diatoms [61]. Together with the most recently published study, which exhibits very similar conclusions (compare our Figs. 2 and Fig. 5B of [49]), our work provides a large number of new experimental data which should be most helpful toward this aim.

4.4 NTA and protein half-life

NTA of proteins is now a well-described modification in Eukaryotes [3, 4, 6, 56]. It was initially discovered more than 50 years ago and its cotranslational nature described in the 1970s (for reviews and references therein, see [62, 63]). The first associated enzymes were described in the late 1980s, although a complete picture is not yet available and the nature of their many substrates was only recently investigated at a large scale [4, 6, 64]. From its early characterization more than two decades ago as an important modification in the cytosol of eukaryotes to its latest fine analyses, its cellular role has been largely discussed [2, 56, 65]. Specific roles of NTA have been described, such as in the case of motor proteins. NTA of tropomyosin N-terminal Met strengthens end-to-end bonds [66, 67] and stabilizes actin filaments and cables [68]. Most recently, a role of NTA in protein stability was proposed to be rather linked to targeting proteins to rapid degradation in the frame of the N-end rule [69]. Hence, NTA could act as a degradation signal (degron). N-terminal-acetylated Ala, Val, Ser, Thr, and Cys residues of proteins could be targeted by the dedicated Doa10 ubiquitin ligase in the yeast *Saccharomyces cerevisiae*. Use of the so-called Ac-N-degron is not a general feature of all cytosolic proteins as it is restricted to dedicated proteins showing N-α-acetylated N-termini to target them to proteasome-dependent degradation. Our study shows that the most unstable stromal proteins rarely possess an NTA in contrast to the most abundant proteins. In this context, two hypotheses can be proposed. Either, NTA – which is post-translational in the case of the stroma – is not effective and rapid enough for this set of very unstable proteins (half-life is <1 h) or NTA is used as a signal important for ensuring improved stability to a stromal protein. Given the importance of N-blocking (N-methylation or NTA) in the most abundant plastid proteins encoded by both the plastid or the nuclear genome [2, 70], we propose that a link between NTA and the increase of protein half-life is most likely. In this case, unlike the Ac-N-degron degradation process which is restricted to a few proteins, the link between NTA and stabilization would apply to the whole stromal proteome. The chloroplast does not display a proteasome but a number of bacterial-type chambered proteases including ClpP, FtsH, Lon and DegP [71–74]. In addition, unlike the cytosol, a majority of the plastid proteins have an N-terminus, which does not depend on NME specificity, extending as a result the spectrum of unusual N-termini. The bacterial ClpAP protease appears to display specificity for such unusual N-termini [75]. To conclude, because of its many differences with the cytosol, plastid proteolysis could well use distinct rules to degrade proteins and control their half-life. In this context, an α-acetylated N-terminus could protect stromal proteins from rapid degradation.

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