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Dual N- and C-Terminal Processing of Citrus Chlorophyllase Precursor Within the Plastid Membranes leads to the Mature Enzyme

Tamar Azoulay-Shemer[1,2], Smadar Harpaz-Saad[1,2,4], Reut Cohen-Peer[1], Anahit Mett[1], Victor Spicer[3], Nicole Lovat[3], Oleg Krokhin[3], Arnon Brand[1,2], David Gidoni[1], Kenneth G. Standing[3], Eliezer E. Goldschmidt[2] and Yoram Eyal[1,*]

[1]Institute of Plant Sciences, The Volcani Center, Agricultural Research Organization, Bet-Dagan 50250, Israel
[2]R. H. Smith Institute of Plant Sciences and Genetics in Agriculture, Faculty of Agriculture, Food, and Environmental Quality Sciences, Hebrew University of Jerusalem, Rehovot 76100, Israel
[3]Manitoba Centre for Proteomics and Systems Biology, University of Manitoba 799 JBRC, 715 McDermot Avenue, Winnipeg, MB, R3E 3P4, Canada
[4]Present address: Department of Biology, University of North Carolina-Chapel Hill, Chapel Hill, NC 27599-3280, USA
*Corresponding author: E-mail, eyalab@volcani.agri.gov.il; Fax, +972-3-9601892
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Chl, the central player in harvesting light energy for photosynthesis, is enzymatically degraded during natural turnover, leaf senescence, fruit ripening or following biotic/abiotic stress induction. The photodynamic properties of Chl and its metabolites call for tight regulation of the catabolic pathway enzymes to avoid accumulation of intermediate breakdown products. Chlorophyllase, the Chl dephytilation enzyme, was previously demonstrated to be an initiator of Chl breakdown when transcriptionally induced to be expressed during ethylene-induced citrus fruit color break or when heterologously expressed in different plant systems. Citrus chlorophyllase was previously shown to be translated as a precursor protein, which is subsequently post-translationally processed to a mature form. We demonstrate that maturation of citrus chlorophyllase involves dual N- and C-terminal processing which appear to be rate-limiting post-translational events when chlorophyllase expression levels are high. The chlorophyllase precursor and intermediate forms were shown to be of transient nature, while the mature form accumulates over time, suggesting that processing may be involved in post-translational regulation of enzyme in vivo function. This notion is further supported by the finding that neither N- nor C-terminal processed domains are essential for chloroplast targeting of the enzyme, and that both processing events occur within the chloroplast membranes. Studies on the processing of chlorophyllase versions truncated at the N- or C-termini or mutated to abolish C-terminal processing suggest that each of the processing events is independent. Dual N- and C-terminal processing, not involving an organellar targeting signal, has rarely been documented in plants and is unique for a plastid protein.

Keywords: Chlorophyll breakdown • Chlorophyllase • Chloroplast • *Citrus limon* • Post-translational regulation • Protein processing.

Abbreviations: Chlase, chlorophyllase; ER, endoplasmic reticulum; GFP, green fluorescent protein; LC/MS, liquid chromatography/mass spectrometry; MALDI-QqTOF, matrix-assisted laser desorption/ionization tandem quadrupole/time-of-flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ORF, open reading frame; PPH, pheophytin pheophorbide hydrolase; PPO, polyphenol oxidase; RT–PCR, reverse transcription–PCR; SGR, stay-green; SSU, small subunit of Rubisco.

The nucleotide sequence reported in this paper has been submitted to GenBank under accession No. FJ174793.

Introduction

Chl is a central player in photosynthesis and its photodynamic properties are the key to the transformation of light energy into chemical energy. However, Chl and its breakdown products are dangerous to the cell when not under strict control, thus its metabolism and particularly the regulation of Chl catabolism are of major importance for the plant cell. Chl breakdown in plants occurs continuously at low levels during the natural turnover of the pigment (Goldschmidt 2001). The breakdown is intense at specific developmental stages such as fruit ripening and leaf senescence (Kräutler 2008), or in response to biotic or abiotic triggers, such as injuries, water shortage or pathogen attack (Benedetti and Arruda 2002, Kariola et al. 2005). The regulation of Chl catabolism is still

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not well understood, and the emerging picture points to a complex regulatory process, which involves two intertwined pathways and two different dephytilation enzymes, pheophorbide hydrolase (PPH) and chlorophyllase (Chlase). The enzyme PPH was recently demonstrated to be the major dephytilation enzyme during Arabidopsis senescence (Schelbert et al. 2009), yet it catalyzes dephytilation of pheophytin but not Chl. The lack of activity on Chl, and the lack of chlorosis when overexpressed (Schelbert et al. 2009), suggest that PPH is not the initiator of Chl breakdown during senescence, a role which may be carried out by the protein SGR, which was shown to be mutated in stay-green mutants of a variety of plant species (Hörtensteiner 2009). Also, SGR was found to be associated with the light-harvesting chlorophyll-binding protein (LHCP) complex and to accelerate Chl breakdown when overexpressed (Jiang et al. 2007, Park et al. 2007, Ren et al. 2007, Aubry et al. 2008). In contrast to PPH, the Chl dephytilation enzyme Chlase from citrus (*Citrus sinensis*) was shown by overexpression experiments in heterologous systems to be an initiator of Chl breakdown within photosynthetically active membranes (Harpaz-Saad et al. 2007), and thus is likely to be involved in Chl breakdown under inductive conditions following biotic or abiotic stress (Benedetti et al. 1998, Tsuchiya et al. 1999, Kariola et al. 2005). This notion is further supported by the finding that Arabidopsis plants silenced for expression of Chlase (*AtCLH1*) fail to degrade free Chl after tissue damage following biotic stress (Kariola et al. 2005). In addition, citrus Chlase gene expression, enzyme accumulation and processing are strongly correlated with ethylene-induced citrus fruit color break (Jacob-Wilk et al. 1999, Azoulay-Shemer et al. 2008), suggesting involvement of the enzyme in ethylene-induced Chl breakdown. However, the involvement of Chlase in Chl breakdown during non-induced fruit color break remains to be addressed.

The degradation pathways of Chl and its photodynamically active breakdown products are expected to be tightly regulated to prevent cell damage, and, thus, expression and function of the pathway enzymes are expected to be regulated at multiple levels. Indeed, we recently provided evidence that citrus Chlase is regulated at both the transcriptional (Jacob-Wilk et al. 1999) and post-translational levels (Harpaz-Saad et al. 2007). The latter may be mediated by N-terminal processing during enzyme maturation, which occurs within the plastid membranes (Harpaz-Saad et al. 2007, Azoulay-Shemer et al. 2008). While it was established that the N-terminal extension of citrus Chlase is not a chloroplast-directing transit peptide (Harpaz-Saad et al. 2007), the questions of its specific role and how its removal affects Chlase in vivo activity remain open.

In this work we delve further into the maturation process of citrus Chlase during ethylene-induced fruit color break and show that citrus Chlase is subject to dual N- and C-terminal, post-translational processing events, which take place within the chloroplast membranes. Both processing events appear to be independent and neither the N- nor the C-termini removed are essential for chloroplast targeting of the enzyme. Similar processing kinetics of the citrus enzyme expressed in a heterologous system (tobacco mesophyll protoplasts) imply an evolutionarily conserved dual processing mechanism. Dual N- and C-terminal processing, which does not involve an N-terminal organelle targeting signal, has rarely been documented in plants and is unique for a plastid protein. The potential significance of the dual processing events as post-translational regulatory steps in Chlase function and Chl degradation is discussed.

Results

Citrus Chlase maturation involves additional processing/post-translational modification events beyond N-terminal processing

We previously demonstrated a strong correlation between Chlase protein expression/maturation and ethylene-induced citrus fruit peel color break (Azoulay-Shemer et al. 2008). In ethylene-treated mature green citrus fruit, Chlase protein is de novo synthesized as a precursor which is subsequently N-terminally processed during enzyme maturation (Trebitsh et al. 1993, Jacob-Wilk et al. 1999, Azoulay-Shemer et al. 2008). Mass spectrometry analysis of the purified Chlase precursor and mature enzyme versions confirmed that the precursor includes the complete N-terminus encoded by the gene open reading frame (ORF), while the mature form is N-terminally processed after amino acids 21, 20 or 19 (Azoulay-Shemer et al. 2008).

In order to improve our ability to monitor the processing kinetics of citrus Chlase during enzyme maturation, we developed recombinant protein markers expressed in *Escherichia coli* corresponding to (i) the citrus Chlase precursor (Rec-Chlase; encoded by the entire ORF) and (ii) the N-terminally processed form (Rec-ChlaseΔN; lacking the N-terminal 21 amino acids encoded by the ORF) (see Marker in **Fig. 1**). Mature green lemon fruits were treated with ethylene (20 μl l^{-1}) or air (Control) at 25°C in the dark for 48 h followed by incubation in the open air. Lemon flavedo tissue was harvested and proteins were extracted, 57 and 96 h from the onset of ethylene treatment. Proteins were separated by extended SDS–PAGE runs aiming for high resolution protein separation, to allow for accurate comparison of the recombinant protein markers with the Chlase protein extracted from the citrus tissue. Immunoblot analysis (**Fig. 1A**) showed that Chlase protein was not detectable in flavedo of mature green non-treated fruit (Control), under standard exposure conditions. Prolonged exposure of the immunoblot membrane revealed low levels of Chlase protein only in the precursor (P) form (35.2 kDa), which has exactly the same electrophoretic mobility as the recombinant full-length Chlase (Rec-Chlase) marker. Mature green fruit treated with ethylene shows a bright greenish-yellow, advanced color break phenotype at 57 h after the onset of ethylene treatment (**Fig. 1B**). In protein samples harvested from the flavedo at this time point, Chlase protein accumulation was observed mainly in the precursor (P) form (35.2 kDa),

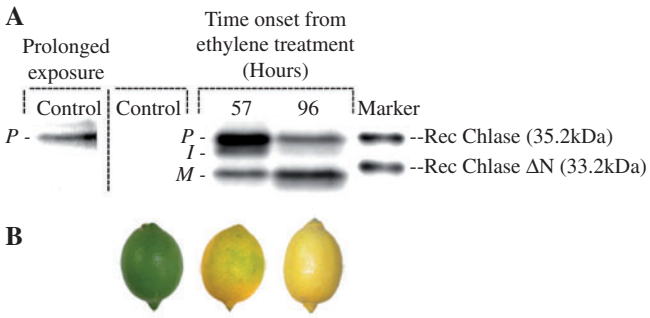


Fig. 1 Kinetics of citrus chlorophyllase (Chlase) accumulation and post-translational maturation during ethylene-induced fruit color break. Mature green lemon fruit were treated with ethylene (20 µl l^{-1}) or with air (Control) at 25°C in the dark for 48 h in a 4 liter sealed container. Following ethylene treatment, lemon fruit were incubated at 25°C in the dark for an additional 9 or 48 h (57 and 96 h from the onset of ethylene treatment). Flavedo tissue from each sample was harvested and total protein was extracted as described in the Materials and methods. (A) Lemon flavedo protein samples from ethylene-treated fruit and control (30 µg protein per lane) were separated by an extended SDS–PAGE run, blotted and decorated with anti-citrus Chlase antibodies for the detection of citrus Chlase versions labeled as: P, precursor; I, intermediate; and M, mature. A mixture of two recombinant citrus Chlase protein versions (0.03 µg protein per lane) serve as size markers: Rec-Chlase, corresponding to the full-length Chlase precursor; and Rec-ChlaseΔN, corresponding to the N-terminally processed Chlase lacking the N-terminal 21 amino acids. The control sample lane was exposed twice, once together with the additional samples (Control) and a second time for a prolonged exposure to enhance detection levels (Prolonged exposure Control). (B) Representative lemon fruit phenotype was monitored and documented at the same time points which were used for flavedo protein extraction.

which has the same electrophoretic mobility as the Rec-Chlase protein marker. The high resolution protein separation revealed two additional faster mobility bands; one band is apparently an intermediate (I) Chlase protein product demonstrating a mobility corresponding to approximately 1 kDa less than the recombinant precursor protein. The second and fastest mobility protein band, corresponding to the mature (M) form of the enzyme, migrated faster than the Rec-ChlaseΔN protein marker, by a difference corresponding to approximately 1 kDa. Mature citrus fruit treated with ethylene for 96 h showed a dramatic, deep yellow, color break phenotype (**Fig. 1B**). In protein samples harvested from the flavedo at this time point, we observed protein bands, corresponding to the precursor (P) and mature (M) Chlase versions, yet the ratio between them shifted in favor of the mature protein. Since the band corresponding to the reproducibly mature citrus Chlase version migrated faster than the Rec-ChlaseΔN protein marker, we deduced that during maturation Chlase is apparently subject to an additional processing or post-translational modification event in addition to the demonstrated N-terminal processing. These results prompted further investigation to elucidate the nature and location of the processing/modification event.

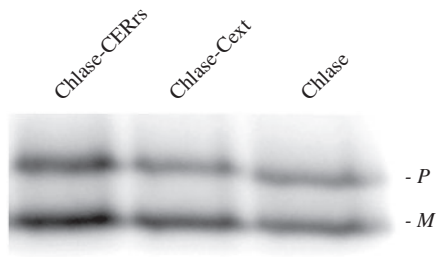


Fig. 2 Transient expression and processing of citrus Chlase fused to a C-terminal extension, or to an ER retention signal. Tobacco leaf protoplasts were transiently transformed with plasmids harboring genes encoding full-length Chlase (Chlase), full-length Chlase C-terminally fused to the ER retention signal-KDEL (Chlase-CERrs) or full-length Chlase C-terminally fused to a four amino acid extension control (KDEV; Chlase-Cext). At 72 h after transformation, protoplasts were harvested and total protein was extracted in USB buffer (Materials and Methods). Protein samples from transiently expressing protoplasts (4.0×10^5 transformed protoplasts extracted in 30 µl of USB per lane) were separated by an extended SDS–PAGE run, blotted and decorated with anti-citrus Chlase antibodies for the detection of citrus Chlase versions labeled as: P, precursor; M, mature.

Expression and processing profile of a C-terminal extension-fused citrus Chlase version suggests the involvement of C-terminal processing in Chlase maturation

In order to shed light on the putative occurrence of an additional processing/modification event during Chlase maturation, beyond N-terminal processing, we studied maturation of modified versions of Chlase using a heterologous tobacco protoplast transient expression system (Harpaz-Saad et al. 2007). The maturation of full-length Chlase expressed in protoplasts was compared with the maturation of a Chlase version fused to an extension of four additional amino acid residues (KDEV) at the C-terminus (Chlase-Cext), resulting in a larger protein. Protein samples from transiently expressing protoplasts were separated by extended SDS–PAGE runs. Immunoblot analysis using citrus Chlase-specific antibodies revealed that maturation of full-length citrus Chlase transiently expressed in tobacco protoplasts (**Fig. 2**) resembles maturation of the protein within its endogenous flavedo tissue (**Fig. 1**). Following 75 h of expression, both precursor (P) and mature (M) Chlase forms were visible.

Maturation of Chlase-Cext, expressed in tobacco protoplasts, was visualized at the 75 h expression time point. Similar to full-length Chlase expression, transient expression of Chlase-Cext resulted in two protein bands corresponding to precursor and mature forms. However, the mobility of the precursor form in Chlase-Cext was, as expected, slightly slower than the precursor (P) form of full-length Chlase. This difference corresponds to the C-terminal addition of four amino acids, which add approximately 0.5 kDa to the protein molecular weight. On the other hand, the mobility of the lower molecular weight protein band of Chlase-Cext, corresponding

to the mature form of the enzyme, was identical to the mobility of the mature (M) form derived from the full-length Chlase. Since the addition of four amino acids at the C-terminus in Chlase-Cext leads to a difference in mobility of the precursor (relative to Chlase), but not the mature protein, we deduced that the mature protein is probably processed at the C-terminus. We thus sought further evidence to confirm C-terminal processing and to locate the processing site.

Mass spectrometry analysis of mature citrus Chlase confirms C-terminal processing

We further addressed the indications for Chlase C-terminal processing by analyzing mature citrus Chlase using mass spectrometry. Mature citrus Chlase protein was purified from peels of ethylene-treated (120 h) lemon fruit by immunoprecipitation followed by SDS–PAGE separation. The purified band of approximately 32 kDa was subjected to proteolysis by trypsin followed by separation and identification of the peptides by liquid chromatography/mass spectrometry (LC/MS) and tandem mass spectrometry (MS/MS) analysis. To explore possible C-terminal processed peptides, arbitrary in silico sequence fragments of citrus Chlase were scanned against the observed mass spectra in search of peptides representing the C-terminal region in general, and semi-tryptic peptides representing the C-terminal end specifically.

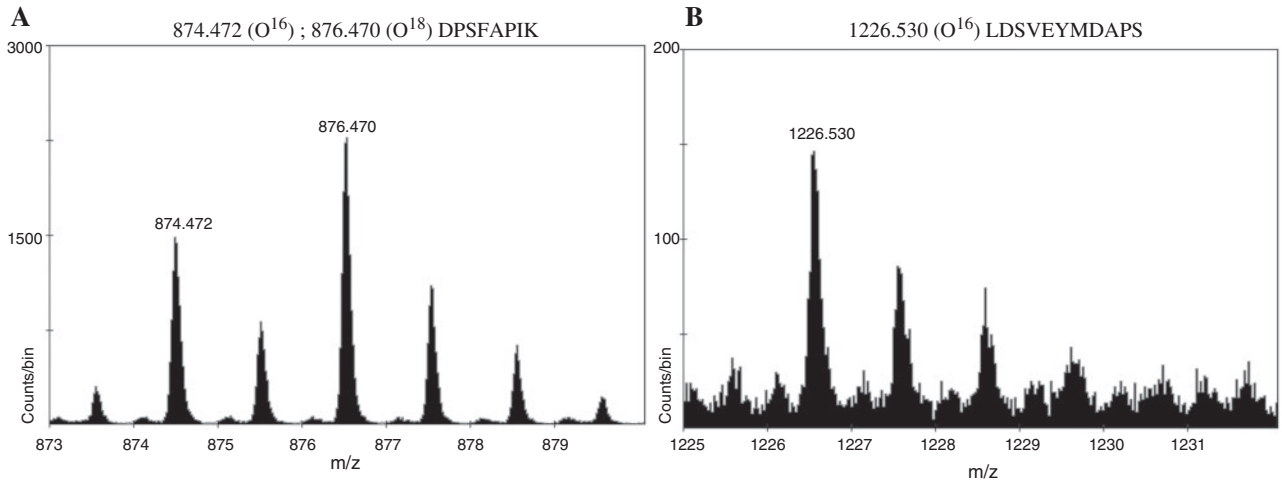
The first experiment involved MS analyses of peptides following tryptic digestion of the mature Chlase protein in a 50/50 solution of ^{18}O water ($H_2{}^{18}O$) and ordinary water ($H_2{}^{16}O$). Two peptide masses corresponding to consecutive C-terminal region peptides were detected; one peptide mass obtained (*m*/*z* 874.472; **Fig. 3A**) corresponds to the C-terminal region tryptic peptide (K)DPSFAPIK containing ^{16}O. The detection of a matching ^{18}O peptide mass (*m*/*z* 876.470; **Fig. 3A**), corresponding to the same peptide, confirms that its end results from the experimental tryptic digest and is not the C-terminal end of the mature Chlase protein. The additional peptide mass obtained (*m*/*z* 1,226.530; **Fig. 3B**) corresponds to a semi-tryptic peptide (K)LDSVEYMDAPS containing ^{16}O, which is immediately downstream of the above-mentioned tryptic peptide in the Chlase sequence. A matching ^{18}O peptide mass corresponding to the same peptide was not detected, suggesting that the non-tryptic end of this peptide represents the true C-terminal end of the mature Chlase generated by in vivo C-terminal processing. Further confirmation of this finding was conducted on a separate experimental sample in which Chlase was subject to a partial trypsin digest in a solution containing ordinary water ($H_2{}^{16}O$). A peptide mass (*m*/*z* 2,081.995) corresponding to a partial trypsin digest, composed of both of the above-mentioned C-terminal region peptides (K)DPSFAPIKLDSVEYMDAPS, was detected and subjected to MS/MS analysis (**Fig. 3C, D**). The retention time for this peptide, which was observed in the spot fraction at 23 min, is in good agreement with the ~23.99 min predicted by the SSRcalc (sequence-specific retention calculator) (Krokhin et al. 2006). MS/MS measurements provided daughter ion pattern data that confirm the peptide sequence identity (**Fig. 3B**) and demonstrate that the mature citrus Chlase protein is C-terminally processed following the amino acid Ser320.

In order to develop further insights regarding the conservation of the Chlase N- and C-terminal processing events in the plant kingdom, we compiled the available data that point to Chlase post-translational processing from plants; the relevant Chlase sequences (from *Citrus limon*, *Chenopodium album* and *Arabidopsis thaliana*) are presented aligned with reference to the suggested processing sites in **Fig. 3E**. The N- and C-terminal processing sites of *C. limon* were demonstrated in Azoulay-Shemer et al. (2008) and in this work, respectively. The N-terminal processing site of *C. album* Chlase (CaCLH) was deduced by comparing the N-terminal protein sequence of the mature protein (Tsuchiya et al. 1997) with the cDNA-encoded ORF (Tsuchiya et al. 1999). Putative N- and C-terminal processing sites of *A. thaliana* Chlase (AtCHL1) are implied by the detection of the semi-tryptic peptides (N-terminal, VVTPAAFEIGSLPTTEIPVDPVENDSTAPPKPVR; C-terminal, LDPSPELEEASG) in a total Arabidopsis proteome analysis (PPBD; the plastid proteome database; ©Klaas J. van Wijk Lab, Cornell University;http://ppdb.tc.cornell.edu/pepseqview.aspx?id=1865). Aligned sequences surrounding the N- and C-terminal processing sites of Chlases from *C. limon*, *A. thaliana* and *C. album* (**Fig. 3E**) call attention to a conserved serine/threonine residue present at the N-terminal processing site. Conserved residues (mostly aspartic acid/glutamic acid) are also evident upstream of the C-terminal processing site. We note that these residues are conserved in functionally characterized Chlases of a variety of plant species (see **Supplementary data**).

Chlase post-translational processing occurs within the chloroplast membrane fraction

We previously demonstrated that N-terminal processing of citrus Chlase precursor transiently expressed in tobacco mesophyll protoplasts occurs within the chloroplast membranes (Harpaz-saad et al. 2007). Due to our present finding, showing that Chlase maturation involves both N- and C-terminal processing, we extended our previous study to include the subcellular localization of the C-terminal processing step. Full-length Chlase or N-terminally deleted Chlase (ChlaseΔN) were transiently expressed in tobacco protoplasts. Protoplasts (72 h post-electroporation) were lysed and intact chloroplasts were isolated on a continuous Percoll gradient. For comprehensive localization, intact chloroplasts were aggressively lysed by freeze/thaw cycles (Materials and Methods), to ensure complete chloroplast lysis, and subjected to fractionation into soluble and membrane fractions by ultracentrifugation. Protein samples were loaded based on equal Chl levels and were separated by SDS–PAGE.

Immunoblot analysis using citrus Chlase-specific antibodies showed that the full-length Chlase version is initially expressed in tobacco protoplasts as an ~35 kDa precursor that is consequently processed (N- and C-terminal processing) to a mature



C

Daughters	b-ion	MH+ calc	m/z observed	Δ(mDa)
DPSFAPIKLDSVEYMDAPS	b19	2081.974	2081.995	21
DPSFAPIKLDSVEYMDA	b17	1879.879	1879.834	-45
DPSFAPIKLDSVEYMD	b16	1808.842	1808.881	39
DPSFAPIKLDSVEYM	b15	1693.815	1693.802	-13
DPSFAPIKLDSVEYM	b15-18	1675.805	1675.792	-13
DPSFAPIKLDSVEY	b14	1562.774	1562.752	-22
DPSFAPIKLDSVE	b13	1399.711	1399.689	-22
DPSFAPIKLDSV	b12-18	1252.658	1252.696	38
DPSFAPIKLD	b10	1084.568	1084.577	9
DPSFAPIKL	b9	969.541	969.536	-5
DPSFAPIKL	b9-17	951.531	951.535	4
DPSFAPI	b7	728.362	728.365	3
DPSFAPI	b7-17	711.335	711.334	-1
DPSFAP	b6	615.278	615.27	-8
DPSFAP	b6-18	597.268	597.3	32
DPSFA	b5	518.225	518.232	7
DPSFA	b5-18	500.215	500.245	30
DPSF	b4	447.188	447.195	7
DPSF	b4-18	429.178	429.16	-18
DPS	b3	300.12	300.136	16

D

Daughters	y-ion	MH+ calc	m/z observed	Δ (mDa)
PSFAPIKLDSVEYMDAPS	y 18	1966.947	1966.946	-1
PSFAPIKLDSVEYMDAPS	y 18- 18	1948.937	1948.97	33
FAPIKLDSVEYMDAPS	y 16- 18	1764.852	1764.827	-25
PIKLDSVEYMDAPS	y 14- 17	1547.73	1547.701	-20
PIKLDSVEYMDAPS	y 14	1564.757	1564.735	-22
KLDSV EYMDAPS	y 12	1354.62	1354.561	-59

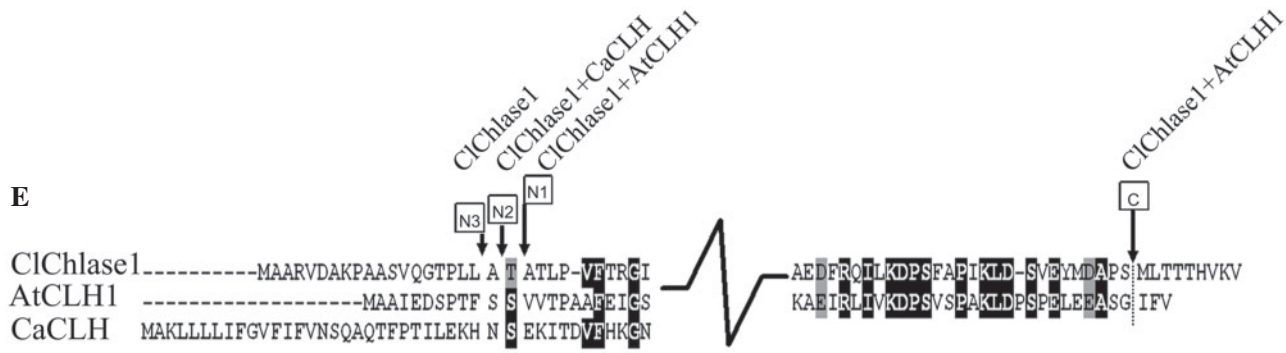


Fig. 3 MS-based characterization of the mature citrus Chlase C-terminal processing site. Mature green lemon fruit were treated with ethylene (20 μl l^{-1}) at 25°C in the dark for 120 h in a 600 liter sealed container. The container was ventilated once every 24 h, followed by injection of fresh ethylene, to maintain CO_2 levels below 1%. Total protein extracted from the flavedo was acetone precipitated, extracted in RIPA buffer and subjected to immunoprecipitation using affinity-purified anti-citrus Chlase antibodies. Immunoprecipitated Chlase versions were separated by SDS–PAGE and visualized by colloidal Coomassie staining. The ~32 kDa protein band corresponding to the mature Chlase form was excised from the gel and subjected to proteolysis by trypsin in the presence of a 50/50 solution of ^{18}O water ($H_2{}^{18}O$) and ordinary water ($H_2{}^{16}O$) (A and B) or only ordinary water ($H_2{}^{16}O$) (C and D). Proteolytic peptides were separated by liquid chromatography, and the precise mass for each peptide was determined by MALDI-QqTOF mass spectrometry. (A) *m/z* spectrum corresponding to the peptide DPSFAPIK. The precise mass of the monoisotopic peaks appears above the peaks. The following multiple peaks correspond to the normal isotopic distribution (i.e. to the inclusion of one, two or three ^{13}C atoms in the peptide molecule). (B) *m/z* spectrum corresponding to the peptide LDSVEYMDAPS. The precise mass of the monoisotopic peak appears above the peak. The following multiple peaks correspond to the normal isotopic distribution (i.e. to the inclusion of one, two or three ^{13}C atoms in the peptide molecule). (C and D) Daughter ions (b and y ions, respectively) observed from MS/MS analysis of the peptide mass corresponding to the semi-tryptic peptide DPSFAPIKLDSVEYMDAPS. Calculated and observed mass are shown for each peptide

Continued

form of ~32 kDa (Fig. 4A), whereas expression of ChlaseΔN gives rise to the N-terminally truncated Chlase version (~33 kDa) that is consequently C-terminally processed to the final mature form of ~32 kDa (Fig. 4B). Identical precursors and processed Chlase products were seen in the whole protoplast samples (P), the purified whole chloroplast samples (C) and chloroplast membrane fractions (CM), but not in the soluble fraction (CS). Extraction of the membrane fraction with buffer containing 200 mM NaCl did not release any of the membrane-associated Chlase versions (data not shown), whereas extraction with buffer containing 0.5% Triton X-100 resulted in complete release of all Chlase forms. The presence of all forms of Chlase in the chloroplast membrane fraction demonstrates that both N- and C-terminal processing events occur with the chloroplast membranes.

The reliability and efficiency of the chloroplast purification protocol was monitored by a similar control experiment in which the green fluorescent protein (GFP) was transiently expressed in tobacco protoplasts in its native form or fused to the chloroplast transit peptide of the small subunit of Rubisco (SSU–GFP) (Fig. 4C). Protoplasts (72 h post-electroporation) were lysed and intact chloroplasts were isolated on a continuous Percoll gradient. Protein samples of whole protoplasts (P) and purified whole chloroplasts (C) were loaded based on equal Chl levels and separated by SDS–PAGE. Immunoblot analysis utilizing GFP-specific antibodies showed that GFP targeted to the chloroplast by a transit peptide (SSU–GFP) is, as expected, detected at comparable levels in protoplasts and chloroplasts (Fig. 4C). In contrast, the native GFP, which is expected to be localized only to the cytosol and nucleus, is indeed present in whole protoplasts but absent from whole chloroplasts. We note a slight reduction in the levels of both Chlase and SSU–GFP in Percoll gradient-purified intact chloroplasts (C) compared with the intact protoplasts (P). We speculate that this difference may result from the possibility that chloroplasts isolated from transiently expressing protoplasts are more fragile than the non-expressing ones, and therefore their representation in the purified intact chloroplast fraction is somewhat reduced.

Citrus Chlase C-terminal processing is not a prerequisite for N-terminal processing

The finding that citrus Chlase maturation involves dual processing provokes questions regarding the order of processing events and the linkage between them. We first questioned whether C-terminal processing is a prerequisite for N-terminal processing of citrus Chlase. C-terminal processing of citrus Chlase was abolished by substituting the sequence surrounding the processing site as follows. We initially introduced substitutions in the *C. sinensis* (orange) Chlase amino acid sequence at positions −2, −1 and +1 (relative to the C-terminal processing site) to three consecutive proline residues (note that proline at −2 naturally exists in lemon Chlase). Versions of full-length Chlase and N-terminally truncated Chlase containing these substitutions (Chlase-mutPPP and ChlaseΔN-mutPPP, respectively) were expressed in tobacco protoplasts for 48 h and protein samples were extracted and separated by extended SDS–PAGE runs, aiming for high resolution protein separation. Immunoblot analysis using citrus Chlase-specific antibodies showed that Chlase-mutPPP was initially expressed as an ~35 kDa precursor (P), similar to the Chlase wild type; however, it was processed to a polypeptide migrating more slowly than the ~32 kDa mature (M) wild-type form (Fig. 5A). ChlaseΔN-mutPPP was initially expressed as an ~33 kDa protein, similar to ChlaseΔN, but was also processed to a polypeptide migrating more slowly than the ~32 kDa mature (M) wild-type form. We deduced that the substitutions introduced in Chlase-mutPPP and ChlaseΔN-mutPPP were not sufficient to abolish processing at the C-terminus, but caused a shift in the processing site downstream from the natural (Ser320/Met321) C-terminal processing site of wild-type citrus Chlase. We therefore introduced three additional amino acid substitutions to proline at amino acid positions +5, +4 and +3 downstream of the cleavage site. The resulting constructs based on the full-length Chlase and N-terminally truncated Chlase (ChlaseXC and ChlaseΔNXC, respectively) contain substitutions for proline at positions −2, −1, +1, +3, +4 and +5 relative to the C-terminal processing site (note that proline at −2 naturally exists in lemon Chlase). Processing of the different Chlase versions was monitored by immunoblot analysis on protein samples extracted from 48 h transiently expressing protoplasts. The results (Fig. 5B) show that the additional proline substitutions completely abolished C-terminal processing in both full-length Chlase (ChlaseXC) and N-terminally truncated Chlase (ChlaseΔNXC) versions; ChlaseXC is synthesized as an ~35 kDa precursor (P) which is processed to an ~33 kDa band, corresponding to N-terminal processing without C-terminal processing. ChlaseΔNXC, which

Fig. 3 Continued
identified at a resolution of <60 ΔmDa. (E) Alignment of demonstrated and inferred N- and C-terminal processing sites in Chlases of the plant kingdom. The N-terminal and C-terminal protein sequences of Chlases, for which data pointing to Chlase post-translational processing are available, are presented: *Citrus limon* (ClChlase1) accession No. FJ174793, *Arabidopsis thaliana* (AtCHL1) accession No. At1g19670 and *Chenopodium album* (CaCLH) accession No. AF134301. The identical amino acids among Chlase sequences are black shaded and the similar amino acids are gray shaded. N- and C-terminal processing sites experimentally determined for Chlase1 are denoted with a black arrow. The three consecutive N-terminal processing sites of Chlase1, denoted as 'N1' (T/A), 'N2' (A/T) and 'N3' (L/A), were demonstrated to occur at a ratio of 63, 33 and 4%, respectively (Azoulay-Shemer et al. 2008). The ClChlase1 C-terminal processing site (this work), located nine amino acids from the C-terminus, is denoted as 'C' (S/M). The deduced N-terminal processing site of CaCLH (Tsuchiya et al. 1997, Tsuchiya et al.1999) is denoted as 'N2' (N/S). Inferred N- and C-terminal processing sites of AtCHL1 (Arabidopsis MS data, PPBD, The plastid proteome database; ©Klaas J. van Wijk Lab, Cornell University; http://ppdb.tc.cornell.edu/pepseqview.aspx?id=1865) are denoted as "N1"(S/V) and "C"(G/I) respectively.

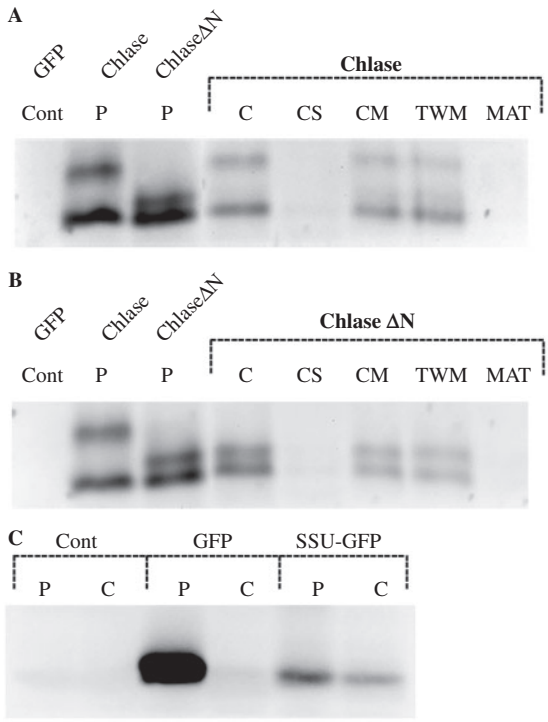


Fig. 4 Chloroplast localization of Chlase forms during enzyme maturation. Tobacco protoplasts were transiently transformed with plasmids harboring genes encoding full-length citrus Chlase (Chlase; A), N-terminally truncated Chlase (ChlaseΔN; B) or the control plasmids harboring the gene encoding GFP (GFP) (A–C), and GFP N-terminally fused to the transit peptide of the Rubisco small subunit (SSU–GFP) (C). Protoplasts were incubated at 25°C in the dark to facilitate expression and were harvested 72 h after transformation. Intact chloroplasts were isolated from lysed protoplasts by Percoll gradient centrifugation and were in turn lysed and fractionated into membrane and soluble fractions. Membrane-associated proteins were washed with buffer containing 200 mM NaCl (data not shown) followed by extraction with buffer containing 0.5% Triton X-100. Proteins were precipitated with acetone and resuspended in USB protein extraction buffer from intact protoplasts (P), intact chloroplasts (C), chloroplast soluble (CS) and membrane (CM) fractions, membranes washed with 0.5% Triton X-100 (TMW), and the remaining membrane fraction after Triton wash (MAT). Proteins were separated by SDS–PAGE (loading based on equal Chl: 6 µg per lane), blotted and decorated with anti-citrus Chlase (A and B) or anti-GFP antibodies (C) for the detection of expressed Chlase versions or GFP, respectively. The control (Cont GFP) lane in the blots decorated with anti-citrus Chlase antibodies (A and B) contains total protein extracted from transformed protoplasts expressing GFP but not expressing any of the citrus Chlase versions. Additionally the control (Cont) lanes in the blots decorated with anti-GFP antibodies (C) contain total protein extracted from protoplasts (P) or intact chloroplasts (C) isolated from non-transformed protoplasts.

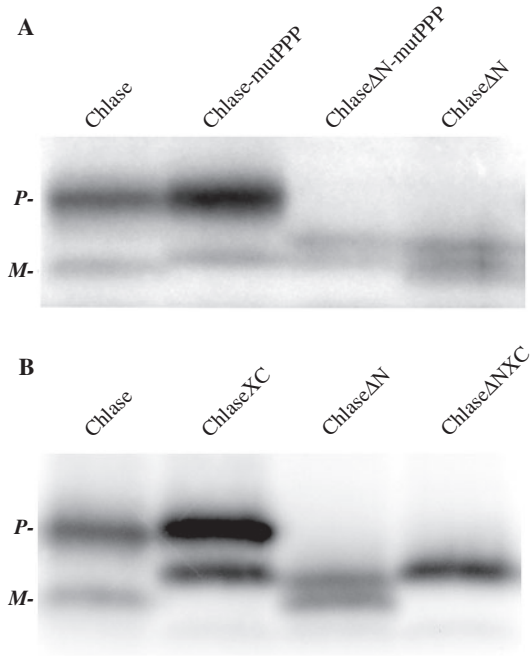


Fig. 5 Processing in citrus Chlase versions containing amino acid substitutions in the C-terminal processing site. Tobacco protoplasts were transiently transformed with plasmids harboring genes encoding either full-length citrus Chlase (Chlase), N-terminally truncated Chlase (ChlaseΔN) or the above Chlase versions containing substitutions in the C-terminal processing site protein region. (A) Plasmids Chlase-mutPPP and ChlaseΔN-mutPPP contain amino acid substitutions to proline at positions +1, −1 and −2 relative to the C-terminal processing site. (B) ChlaseXC and ChlaseΔNXC contain amino acid substitutions to proline at positions +5, +4, +3, +1, −1 and −2, relative to the C-terminal processing site. At 48 h after transformation protoplasts were harvested and total protein was extracted in USB buffer. Protein samples from transiently expressing protoplasts (4.0×10^5 transformed protoplasts extracted in 30 µl of USB per lane) were separated by an extended SDS–PAGE run, blotted and decorated with anti-citrus Chlase antibodies for the detection of citrus Chlase versions labeled as: P, precursor; M, mature.

lacks the N-terminal 21 amino acids, is synthesized as an ~33 kDa band, but, unlike ChlaseΔN is not processed at all. We note that the mobility of the precursor bands of Chlase-mutPPP, ChlaseΔN-mutPPP, ChlaseXC and ChlaseΔNXC is slightly slower relative to their wild-type counterparts (Chlase and ChlaseΔN) (**Fig. 5A, B**). This mobility shift is probably due the substitution of three/six amino acids to proline. This notion is further supported by the increased mobility shift in ChlaseXC and ChlaseΔNXC, which contain six proline substitutions, relative to Chlase-mutPPP and ChlaseΔN-mutPPP that contain only three proline substitutions.

We further questioned whether the presence of an N-terminus is essential for C-terminal processing and whether the presence of a C-terminus is essential for N-terminal processing. Constructs designed for expression of Chlase versions corresponding to N-terminally processed citrus Chlase (ChlaseΔN) and C-terminally processed citrus Chlase (ChlaseΔC) were expressed in tobacco protoplasts and proteins were extracted. Immunoblot analysis demonstrated that N-terminally truncated Chlase (ChlaseΔN) is C-terminally processed to obtain the mature Chlase (**Figs. 4, 5**), whereas C-terminally

truncated Chlase is N-terminally processed to obtain the mature Chlase (Fig. 6). Thus, we concluded that C-terminal processing is not a prerequisite for N-terminal processing and that the presence of the N- or C- termini is not essential for in vivo processing of the opposite termini.

The Chlase C-terminal processed domain is not essential for protein import into the chloroplast

We previously demonstrated that the processed N-terminus is not involved in import into the plastid (Harpaz-Saad et al. 2007). Additionally, we provided evidence (this work) which show that both N- and C-terminal processing events occur within the plastid membranes. In order to examine whether the C-terminal domain is essential for citrus Chlase import into the chloroplast, we generated a construct for expression of C-terminally truncated Chlase that lacks the C-terminal nine amino acids (i.e. corresponding to the C-terminal processing; ChlaseΔC). Full-length Chlase, N-terminally-truncated Chlase (ChlaseΔN), C-terminally truncated Chlase (ChlaseΔC) and GFP (cytoplasmic) control constructs were transiently expressed in tobacco protoplasts for 48 h. Intact chloroplasts from lysed protoplasts were obtained via a Percoll cushion. Total protoplast and isolated chloroplast samples were loaded based on equal Chl levels and were separated together with the citrus Chlase recombinant markers (Rec-Chlase and Rec-ChlaseΔN) by extended SDS–PAGE. Immunoblot analysis shows that ChlaseΔC is initially expressed as an ~34 kDa polypeptide, approximately 1 kDa less than the full-length Chlase recombinant marker (Rec-Chlase). This ~34 kDa ChlaseΔC precursor was processed to an ~32 kDa polypeptide, matching the N- and C-terminally processed fully mature Chlase (Fig. 6). Precursor and mature versions of ChlaseΔC as well as the wild-type Chlase were present in both total protoplast and isolated chloroplast samples, demonstrating that the citrus Chlase C-terminal processed domain is not essential for protein import into the chloroplast.

We further studied whether abolishing C-terminal processing affects chloroplast import. ChlaseXC (citrus Chlase containing six proline substitutions at positions −2, −1, +1, +3, +4 and +5 relative to the C-terminal processing site) was transiently expressed in tobacco protoplasts, as described above, and total protoplast and isolated chloroplast protein samples were extracted, separated by SDS–PAGE and analyzed by immunoblot (Fig. 6). Transient expression of ChlaseXC gave rise to a precursor band, which migrated slightly more slowly than the full-length Chlase precursor form, apparently due to the amino acid substitutions (as previously shown in Fig. 5B). The ChlaseXC precursor was processed to a form co-migrating with the N-terminally truncated citrus Chlase form (Rec-ChlaseΔN marker), but not further processed to the mature form. Precursor and mature versions of ChlaseXC were present in both total protoplast and isolated chloroplast samples, demonstrating that abolishing C-terminal processing, and the substitutions introduced, do not affect Chlase chloroplast import.

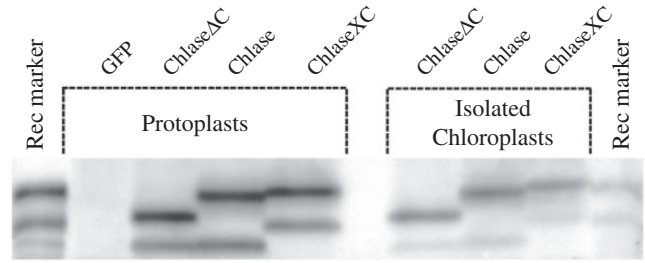


Fig. 6 Chloroplast localization of processing events in Chlase versions containing C-terminal deletion or substitutions. Tobacco protoplasts were transiently transformed with plasmids harboring genes encoding full-length Chlase (Chlase), C-terminal truncated Chlase (ChlaseΔC) or with full-length Chlase containing C-terminal amino acid substitutions that abolish C-terminal processing (ChlaseXC; amino acid substitutions to proline at positions +5, +4, +3, +1, −1 and −2 relative to the C-terminal processing site). Protoplasts were incubated at 25°C in the dark to facilitate expression and were harvested 48 h after transformation. Intact chloroplasts were isolated from lysed protoplasts by Percoll gradient centrifugation. Proteins from intact protoplasts and intact chloroplasts were precipitated with acetone and resuspended in USB protein extraction buffer. Proteins were separated by SDS–PAGE (loading based on equal Chl: 20 μg per lane), blotted and decorated with anti-citrus Chlase antibodies for the detection of expressed Chlase versions. The control (GFP) lane in the blots decorated with anti-citrus Chlase antibodies contains total protein extracted from transformed protoplasts expressing GFP but not expressing any of the citrus Chlase versions. Rec marker is a precise Chlase version size marker composed of a mixture of two recombinant citrus Chlase protein versions: Rec-Chlase, corresponding to the full-length Chlase precursor; and Rec-ChlaseΔN, corresponding to the N-terminally processed Chlase, lacking the N-terminal 21 amino acids.

Targeting of citrus Chlase to the chloroplast is still an enigma

The vast majority of nuclear-encoded chloroplast-localized proteins are targeted to the chloroplast by an N-terminal transit peptide and via the well characterized chloroplast Tic/Toc import mechanism (Jarvis 2008). However, the evidence we present demonstrates that citrus Chlase is targeted to the chloroplast via a non-canonical mechanism/route, not involving the N- or C-terminus (Harpaz-Saad et al. 2007, and Fig. 6, respectively). One alternative targeting route to the chloroplast has been demonstrated to involve transport of nuclear-encoded chloroplast proteins via the endoplasmic reticuloum (ER) to the Golgi followed by transport to the chloroplast by vesicles (Radhamony and Theg 2006). This pathway appears to be specific for glycoproteins, such as carbonic anhydrase (Villarejo et al. 2005), nucleotide pyrophosphate/phosphodiesterase (Nanjo et al. 2006) and α-amylase (Kitajima et al. 2009). Since preliminary data suggested that Chlase (from *Chenopodium*) may be a glycoprotein (Tsuchiya et al. 1997, Tsuchiya et al. 1999), we examined the possibility that Chlase utilizes the ER–Golgi pathway for chloroplast targeting. We therefore designed a construct for transient expression of Chlase fused

C-terminally to the ER retention signal (KDEL) (Chlase-CERrs) based on the rationale that if Chlase is transported via the ER, fusion of the KDEL retention signal would result in ER retention, thus preventing chloroplast import. A control vector was designed for transient expression of Chlase fused C-terminally to a similar sequence that is inactive in ER retention (KDEV) (Chlase-Cext). Protein samples from transiently expressing protoplasts (72 h post electroporation) were separated by extended SDS–PAGE runs followed by immunoblot analysis. The results (**Fig. 2**) show an identical expression/maturation pattern for wild-type Chlase, Chlase-CERrs (C-terminal ER retention signal fusion) and Chlase-Cext (C-terminal control fusion). All Chlase versions were initially expressed as a precursor (P) form (~35 kDa for the wild-type Chlase and slightly larger for Chlase-CERrs and Chlase-Cext) and were subsequently processed to the mature (M) form (~32 kDa). Since we already established that N- and C-terminal processing occur within the chloroplast, it was clear the Chlase-CERrs was not retained in the ER. We deduced that Chlase is targeted to the chloroplast via an alternative route, probably not involving the ER.

Discussion

In this work we demonstrated that chloroplast-localized citrus Chlase is subjected to dual post-translational processing, at both the N- and C-termini. Citrus Chlase endogenous expression in citrus fruit or heterologous expression in tobacco cells follows a similar kinetic pattern of processing, culminating in the accumulation of a dual-processed mature protein. Both forms of the Chlase protein (precursor and mature) were detected in the chloroplast membrane fraction of citrus Chlase-expressing tobacco cells, suggesting that both N- and C-terminal processing events occur within the chloroplast membranes. Yet, it remains to be determined whether the different Chlase forms are located in the same chloroplast membrane or rather segregate between different chloroplast membranes. Beyond the common plastidic localization, N- and C-terminal processing events appear to be independent of each other; N-terminal processing occurs in Chlase versions lacking the C-terminus, and C-terminal processing occurs in Chlase versions lacking the N-terminus. Furthermore, amino acid substitutions abolishing C-terminal processing do not affect N-terminal processing, providing further support for the notion that these are two independent post-translational processing events.

The data we present here, as well as previously published results (Harpaz-Saad et al. 2007, Azoulay-Shemer et al. 2008), demonstrate that citrus Chlase is localized to the chloroplast. The vast majority of nucleus-encoded chloroplast proteins are directed to the chloroplast via a mechanism involving an N-terminal transit peptide, which typically undergoes cleavage upon entry into the chloroplast (reviewed in Fuks and Schnell 1997). Protein domains in the C-terminus of some chloroplast proteins have also been implicated in influencing import and targeting competence to the chloroplast (Ko and Ko 1992). Yet in the case of citrus Chlase, we previously demonstrated that the N-terminus does not function as a chloroplast-targeting transit peptide (Harpaz-Saad et al. 2007). Furthermore, our current work demonstrates that the C-terminus is also not essential for chloroplast targeting. Thus, the accumulating data suggest that Chlase is targeted to the chloroplast via a non-canonical, alternative, import pathway, as previously suggested for other plastidic proteins (Miras et al. 2002, Nada and Soll 2004). We note that recent work has demonstrated the existence of an alternative protein targeting pathway to the chloroplast via the ER and Golgi (Jarvis 2008), apparently restricted to glycoproteins such as carbonic anhydrase (Villarejo et al. 2005, Radhamony and Theg 2006), nucleotide pyrophosphatase/phosphodiesterase 1 (Nanjo et al. 2006) and α-amylase (Kitajima et al. 2009). Since some works suggest that Chlase may be a glycoprotein (Tsuchiya et al. 1997, Tsuchiya et al. 1999), we were encouraged to examine whether citrus Chlase utilizes the ER–Golgi–chloroplast pathway. The results demonstrate that fusion of a C-terminal ER retention signal did not affect chloroplast localization and processing of Chlase, and therefore it is not likely that Chlase is targeted to the chloroplast via the ER. Thus, the mechanism employed for targeting of citrus Chlase to the chloroplast remains an enigma.

Dual N- and C-terminal processing of Chlase appears to be an evolutionarily conserved mechanism in the plant kingdom, since citrus Chlase follows similar processing kinetics in the endogenous citrus tissue and when expressed in tobacco cells. Furthermore, detection of semi-tryptic peptides (N- and C-terminal) of Chlase in a proteomic analysis of Arabidopsis seedlings together with N-terminal protein sequencing of the mature *C. album* Chlase (CaCLH) (Tsuchiya et al. 1997, Tsuchiya et al. 1999) reveal that analogous post-translational processing events probably also occur in Arabidopsis Chlase (AtCHL1) and *Chenopodium* Chlase (CaCLH1) (**Fig. 3E**). We note that the sequence of the N- and C-termini of functionally characterized Chlases is relatively not conserved (**Fig. 3E** and **Supplementary data**), and thus conserved amino acids stand out. At the N-terminus, a conserved serine/threonine residue is prominent flanking the processing site. We did not detect conserved sequences at or downstream of the processing site at the C-terminus, yet several conserved residues, mainly aspartic/glutamic acid, are located upstream of the C-terminal processing site. The significance of these conserved residues remains to be experimentally studied.

Finally, dual N- and C-terminal processing of proteins has rarely been documented in plants and only once for a plastidic protein—polyphenol oxidase (PPO), in which the N-terminal cleaved domain functions as a transit peptide while its C-terminal cleaved domain is proposed to be involved in enzyme regulation (Rathjen and Robinson 1992, Gerdemann et al. 2002, Marusek et al. 2006). As in the case of PPO, most N-terminal processed sequences are targeting signals, but there are some examples for dual N- and C-terminal processing, in

which both domains are involved in enzyme activity regulation, for instance the vacuolar protein RD21(Yamada et al. 2001). Similarly, citrus Chlase appears to be unique as a dual-processed chloroplast enzyme, since we demonstrate that both N- and C-terminus processed domains are not involved in import to the plastid (Harpaz-Saad et al. 2007, and this work, respectively). We note in this context that in the endogenous and heterologous systems studied, the precursor and intermediate Chlase forms appear to be transient, while the mature protein accumulates, suggesting that Chlase processing events are involved in enzyme maturation and not as steps towards protein degradation. Nevertheless, the precise roles of the N- and C-terminal processing events remain unsolved. Possible roles that can be envisaged include: (i) regulating Chlase subplastidic localization, which may affect interaction of the enzyme with its substrate; (ii) removal of regulatory domains which affect Chlase activity—a mechanism of this nature has been described for the enzyme catechol oxidase/PPO (Gerdemann et al. 2002); (iii) exposing N- and/or C-termini to interactions with other molecules essential for function, e.g. processing of the C-terminus of D1 exposes the binding site for a manganese atom of the Mn_4Ca cluster, which is responsible for the water oxidation chemistry in PSII (Nixon et al. 1992, Debus 2005); and (iv) exposing protein domains or affecting their structure may also facilitate protein complex formation. We note in this context that Chlase has been suggested to function as part of a multisubunit or multiprotein complex (Trebitsh et al. 1993, Arkus et al. 2005). Indeed, dimerization and complex assembly can function as mechanisms regulating enzyme activity (Sassoon et al. 1999, Hendriks et al. 2003, Huesgen et al. 2007).

Future work directed to understanding the physiological roles of Chlase N- and C-terminal processing, the proteases involved and the mechanisms of chloroplast and intraplastidic compartment targeting appears to be key to understanding the post-translational regulation of Chlase in vivo activity and the regulatory implications for Chl breakdown.

Materials and Methods

Plant material and treatment

For Chlase kinetics analysis, mature green lemon fruit (*C. limon*) were treated with ethylene (20 µl l^{-1}) or with air (control sample) at 25°C in the dark for 48 h in a 4 liter sealed container. Following ethylene treatment, the container was ventilated and lemon samples were incubated at 25°C in the dark for am additional 9 or 48 h (57 and 96 h from the onset of ethylene treatment). Samples of the flavedo (the outer, pigmented part of the fruit peel) were peeled, frozen in liquid nitrogen, ground to a powder and used for protein extraction.

For Chlase immunoprecipitation and total RNA extraction, mature green lemon fruit were treated with ethylene (20 µl l^{-1}) at 25°C in the dark for 48 or 120 h in a 600 liter sealed container. The container was ventilated once every 24 h, followed by injection of fresh ethylene to maintain CO_2 levels below 1%. Flavedo samples for RNA extraction were frozen in liquid nitrogen and stored frozen until use (−80°C). Flavedo samples for immunoprecipitation were used fresh.

Construction of Chlase and GFP expression plasmids, and transient expression in tobacco protoplasts

Constructs for transient protoplast transformation were prepared in the cloning vector pUC-19. A basic cloning plasmid (p35S vector) was created by cloning the 35S promoter upstream of the tobacco etch virus (TEV) translation enhancer (Gallie et al. 1995), a multicloning site containing *Nco*I and *Sal*I restriction sites (5′ to 3′ orientation, respectively) and the 35S terminator.

Next, the following genes were subcloned into the p35S vector, using the *Nco*I and *Sal*I restriction sites. (i) GFP plasmid: the gene encoding enhanced GFP (GFP-S65T) (Heim et al. 1995); (ii) SSU–GFP plasmid: GFP-S65T N-terminally fused to the sequence encoding the 57 amino acid chloroplast transit peptide of the Rubisco small subunit (SSU) (accession No. P00869); (iii) Chlase plasmid: full-length Chlase version (990 bp ORF); (iv) ChlaseΔN plasmid: N-terminally truncated Chlase version (924 bp ORF, lacking the first 21 codons but supplemented with an ATG initiator codon); (v) ChlaseΔC: C-terminally truncated Chlase version (960 bp ORF, lacking the C-terminal end nine codons); (vi) Chlase-CERrs: C-terminally fused Chlase version, designed to include the ER retention signal (KDEL); (vii) Chlase-Cext: C-terminally fused Chlase version, designed to include extension of four additional amino acid residues (KDEV) at the C-terminus. Four additional constructs were prepared on the backbone sequence of full-length (Chlase) or N-terminally deleted citrus Chlase versions (ChlaseΔN); the constructs (viii) Chlase-mutPPP and (ix) ChlaseΔN-mutPPP include conversion of the amino acids at positions +1, −1 and −2 from the C-terminal processing site to proline; and the constructs (x) ChlaseXC and (xi) ChlaseΔNXC include conversion of the amino acids at positions +5, +4, +3, +1, −1 and −2 from the C-terminal processing site to proline.

All Chlase versions are based on the *C. sinensis* Chlase gene sequence (CsChlase1; accession No. AAF59834).

Mesophyll protoplasts were isolated from tobacco (*Nicotiana tabacum* cv Samsun NN) plants grown under sterile conditions (Draper et al. 1988). Electroporation of 4×10^5 protoplasts per construct was performed in pre-chilled electroporation medium (Fromm et al. 1985) using 5 µg of plasmid DNA, 15 µg of calf thymus DNA and 0.5 ml of electroporation solution. After electroporation, the protoplasts were transferred into growth medium and incubated in darkness at 27°C until harvesting. Protoplasts were harvested by centrifugation for 5 min at 100×*g*, 20°C. Medium was discarded and protoplast pellets (8×10^5 protoplasts) were homogenized by vigorous vortexing in lysis buffer (20 mM Tris–HCl, pH 7.5) followed by protein extraction.

Protein extraction

Proteins were acetone precipitated from protoplasts or flavedo by addition of 5 vols. (w/v) of cold acetone (−20°C) followed by overnight incubation at −20°C. Samples were then centrifuged for 10 min at 15,000×*g*, 4°C, following by air drying of the pellet.

Acetone-precipitated protein samples (flavedo dry weight, 45 mg; protoplasts, 8×10^5 cells) were homogenized and incubated in (flavedo, 450 µl; protoplasts, 60 µl) USB buffer (20 mM Tris–HCl, pH 7.5, 8 M urea, 4.5% SDS and 1 M β-mercaptoethanol) for 1 h at 25°C. Extracts were centrifuged for 15 min at 15,000×*g* at 25°C, the supernatant was transferred to a new tube, and the centrifugation step was repeated twice more in order to obtain a clear soluble protein solution. Equal amounts of total protein from each sample were separated by SDS–PAGE (12%) and subjected to protein gel blot analysis as described below.

Isolation of chloroplasts and fractionation

For preparation of chloroplasts for fractionation, a total of 3.5×10^6 protoplasts were pooled from transformations of each construct. Protoplasts were lysed for 30 min on ice in protoplast lysis buffer (50 mM HEPES, pH 8, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.33 M sorbitol, 1 mM ascorbic acid and 20 µg ml bovine serum albumin). Intact chloroplasts were isolated on a continuous Percoll gradient according to Adam and Hoffman (1993) and Levy and Adam (1995). Isolated intact chloroplasts were aggressively lysed in chloroplast lysis buffer (20 mM Tris–HCl, pH 7.5) by three freeze/thaw cycles in liquid nitrogen, each followed by vigorous vortexing on the partially thawed samples. Membrane and soluble fractions were separated by centrifugation at 100,000×*g* (rotor SW-50; Beckman) for 1 h. The membrane pellet was extracted with 20 mM Tris–HCl, pH 7.5, and 200 mM NaCl for 10 min and centrifuged at 100,000×*g* for 1 h. The pellet was then extracted with 20 mM Tris–HCl, pH 7.5, and 0.5% Triton X-100 for 10 min and centrifuged at 100,000×*g* for 1 h. The washed pellet was resuspended in chloroplast lysis buffer. Samples were brought to a final volume of 100 µl, and 5 vols. of cold acetone were added. Precipitated proteins were extracted in USB protein extraction buffer (as described previously). Chl concentration was determined according to Arnon (1949) and used to calculate the protein equivalent to 6 µg of Chl from each fraction for SDS–PAGE.

Expression of recombinant full-length and N-terminal truncated Chlase versions in bacteria

A precise protein marker corresponding to the full-length (Rec-Chlase) and the N-terminally processed citrus Chlase (Rec-ChlaseΔN) was produced using the PET-28a expression vector system (Novagen).

The citrus Chlase cDNAs encoding the full-length (990 bp ORF) and the N-terminally truncated (924 bp ORF) citrus Chlase versions were excised by *Nco*I and *Sal*I digestion from the p35S-Chlase and p35S-ChlaseΔN plasmids, respectively. The excised inserts were then ligated into the *Nco*I and *Sal*I sites of the PET-28a vector. These plasmids were transformed into *E. coli* BL21 LysE cells (Novagen). Cells carrying the citrus Chlase full-length (Rec-Chlase) and the N-terminally truncated citrus Chlase (Rec-ChlaseΔN) expression vectors were induced for recombinant protein expression by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and were grown at 37°C for 3 h. A 3 ml aliquot of bacterial cells was then harvested by centrifugation at 4,000×*g* for 10 min. The pellet was resuspended in 1 ml of USB extraction buffer (20 mM Tris–HCl, pH 7.5, 8 M urea, 4.5% SDS and 1 M β-mercaptoethanol) for 1 h at 25°C. Extracts were centrifuged for 15 min at 15,000×*g* at 25°C, the supernatant was transferred to a new tube. A 1:1 ratio mixture of both lysates, diluted 1:200 in USB buffer, was used as the citrus recombinant marker stock (Rec-Chlase; 35.2 kDa + Rec-ChlaseΔN; 32.2 kDa). A 20 µl aliquot from the marker stock was used for each lane of the SDS–polyacrylamide gels (12%).

Protein immunoblot analysis

Proteins separated by SDS–PAGE were transferred onto nitrocellulose membranes (Schleicher & Schuell) as described (Page and Thorpe 1996). Membranes were blocked and incubated overnight with mouse anti-citrus Chlase polyclonal antibodies diluted 1:1,000 (Harpaz-Saad et al. 2007) or with mouse anti-GFP monoclonal antibodies (Roche Diagnostics) diluted 1:1,000 for 1.5 h at room temperature. Immunoblots were developed using the SuperSignal West Dura or Femto Substrate (Pierce) after incubation for 1 h with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:10,000 (Jackson ImmunoResearch Laboratories Inc.). Decorated immunoblots were visualized in a FluorChem 8800 imaging system (Alpha Innotech).

Immunoprecipitation and gel purification of Chlase versions

Acetone powder samples (3 g) of ethylene-treated (120 h) lemon fruit flavedo were homogenized and incubated in 30 ml of RIPA buffer (20 mM Tris–HCl pH 7.2, 100 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate) containing complete EDTA-free protease inhibitor cocktail (Roche) in a rotator at 4°C for 2 h. Extracts were centrifuged for 15 min at 15,000×*g* at 4°C, the supernatant was transferred to a new tube and the centrifugation step was repeated twice more in order to obtain a clear soluble protein solution. The protein extract was then incubated with (40 µl) anti-Chlase antibodies (raised in mice; Harpaz-Saad et al. 2007) in a rotator at 4°C for 12–15 h.

Protein A–agarose beads (10 µl), prepared as described by the manufacturer (Sigma), were added and rotational incubation was continued for an additional 4 h. The immunoprecipitated complex was collected by centrifugation at 420×*g*, 4°C for 2 min, and the supernatant was aspirated. The agarose beads were washed three times with ice-cold RIPA buffer. Immunoprecipitated protein was eluted in 30 µl of USB buffer (20 mM Tris–HCl, pH 7.5, 8 M urea, 4.5% SDS and 1 M β-mercaptoethanol)

by incubation for 15 min at 25°C, followed by centrifugation for 5 min at 420×*g*, 20°C. The protein containing supernatant was transferred to a new tube.

In order to separate and isolate the various versions of Chlase protein, immunoprecipitated protein samples were separated by 12% SDS–PAGE as described (Page and Thorpe 1996). Gels were then stained in colloidal Coomassie under gentle shaking at 25°C as follows: gels were initially fixed by incubation for 1 h in fixation solution (50% ethanol; 12% acetic acid) and were then transferred to freshly made staining solution [10% (w/v) ammonium sulfate; 10% phosphoric acid; 0.12% colloidal Coomassie Brilliant Blue G250 (Fluka); 20% methanol, in HPLC grade double-distilled water]. Gels were stained for 16–20 h, washed twice for 20 min in de-staining solution (20% ethanol), followed by washing with double-distilled water for 20 min. Protein gel bands of the mature Chlase version were carefully excised and submitted to analysis by mass spectrometry as detailed below.

Mass spectrometric analyses of protein bands

Proteins in excised gel slices were first subjected to a tryptic in situ overnight digest, in the presence of a 50/50 solution of ^{18}O water ($H_2{}^{18}O$) (Isotec) and ordinary water ($H_2{}^{16}O$) or only ordinary water as described (Shevchenko et al. 1997, Stewart et al. 2001, Yao et al. 2003). In preliminary examinations, digests were measured directly (without prior reversed-phase HPLC fractionation) in a matrix-assisted laser desorption/ionization tandem quadrupole/time-of-flight (MALDI-QqTOF) mass spectrometer (Loboda et al. 2000). For more detailed analyses, the proteolyzed fragments were separated into forty 1 min fractions by reversed-phase HPLC and deposited onto a MALDI target by a robot previously constructed in the Manitoba laboratory (Krokhin et al. 2004). The mass-to-charge spectrum for each spot was then acquired in the same TOF instrument. The candidate protein was initially identified using SMART, a software tool for retention time-based peptide mass fingerprinting developed at the University of Manitoba (Krokhin et al. 2006).

To explore possible C-terminal peptides, we generated in silico arbitrary sequence fragments from the citrus Chlase-deduced amino acid sequence and scanned them against the observed mass spectra, allowing up to 60 mDa variation in mass and ±5 min in retention time.

Isolation and cloning of the cDNA encoding Chlase from citrus limon

Citrus limon Chlase was amplified by reverse transcription–PCR (RT–PCR) and PCR amplifications as follows. Total RNA (5 µg) from 48 h ethylene-induced lemon flavedo was prepared as previously described by Jacob-Wilk et al. (1999) and used as a template for single strand cDNA synthesis using the tailed oligo(dT) primer (5′-GTTTTCCCAGTCACGACGTTTTTTTT TTTTTTT-3′) and MMLV reverse transcriptase (Promega Corporation). The full sequence of *C. limon* Chlase was PCR amplified, using the cDNA template, the proofreading enzyme Pyrobest DNA polymerase (TAKARA) and the following primers: (i) the gene-specific 5′ end primer 5′-GCGGAAAAA AAATCACGATAATG-3′, which was synthesized on the basis of the homologous region of the Chlase 5′-untranslated region sequences in *Citrus medica* and *C. sinensis*; and (ii) the tail primer 3′ end, which is homologous to the tail of the reverse transcription primer 5′-GTTTTCCCAGTCACGACG-3′. The 990 bp amplified cDNA band was purified by gel electrophoresis, cloned into pGEM-T easy vector (Promega) and sequenced (*ClChlase1*; accession No. FJ174793).

Supplementary data

Supplementary data are available at PCP online.

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