Citrus Chlorophyllase Dynamics at Ethylene-Induced Fruit Color-Break: A Study of Chlorophyllase Expression, Posttranslational Processing Kinetics, and in Situ Intracellular Localization\textsuperscript{1[OA]}

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Fruit color-break is the visual manifestation of the developmentally regulated transition of chloroplasts to chromoplasts during fruit ripening and often involves biosynthesis of copious amounts of carotenoids concomitant with massive breakdown of chlorophyll. Regulation of chlorophyll breakdown at different physiological and developmental stages of the plant life cycle, particularly at fruit color-break, is still not well understood. Here, we present the dynamics of native chlorophyllase (Chlase) and chlorophyll breakdown in lemon (Citrus limon) fruit during ethylene-induced color-break. We show, using in situ immunofluorescence on ethylene-treated fruit peel (flavedo) tissue, that citrus Chlase is located in the plastid, in contrast to recent reports suggesting cytoplasmic localization of Arabidopsis (Arabidopsis thaliana) Chlases. At the intra-organellar level, Chlase signal was found to overlap mostly with chlorophyll fluorescence, suggesting association of most of the Chlase protein with the photosynthetic membranes. Confocal microscopy analysis showed that the kinetics of chlorophyll breakdown was not uniform in the flavedo cells. Chlorophyll quantity at the cellular level was negatively correlated with plastid Chlase accumulation; plastids with reduced chlorophyll content were found by in situ immunofluorescence to contain significant levels of Chlase, while plastids containing still-intact chlorophyll lacked any Chlase signal. Immunoblot and protein-mass spectrometry analyses were used to demonstrate that citrus Chlase initially accumulates as an approximately 35-kD precursor, which is subsequently N-terminally processed to approximately 33-kD mature forms by cleavage at either of three consecutive amino acid positions. Chlase plastid localization, expression kinetics, and the negative correlation with chlorophyll levels support the central role of the enzyme in chlorophyll breakdown during citrus fruit color-break.

Color-break and the enhanced biosynthesis of aroma compounds toward fruit ripening are strategies developed by plants to attract seed dispersal agents to fruits that contain fully mature seeds. In citrus, developmentally regulated fruit color-break involves accumulation of copious amounts of carotenoid pigments concomitant with chlorophyll breakdown (Masaya et al., 2004). Both metabolic processes occur within the plastids of the flavedo tissue (the pigmented outer part of the peel) and are the visual manifestation of the transformation of the flavedo photosynthetically active chloroplasts into chromoplasts (Thomson and Whatley, 1980; Goldschmidt, 1988; Kobayashi, 1991).

The chlorophyll molecule is a central player in harvesting light energy channeled for photosynthesis in leaves, yet it likely assumes other tissue-specific roles in the fruit peel, such as providing green pigmentation that camouflages the fruit until the seeds are mature. Indeed, the photosynthetic role in fruit appears to be limited, as photosynthetic levels of fruit peel chloroplasts have been documented to be quite low and account for only a few percent of the sugar accumulating in the fruit (Piechulla et al., 1987). Regulation of chlorophyll breakdown at the different physiological and developmental stages of the plant life cycle and particularly at fruit color-break is still not well understood. Tight regulation of the breakdown pathway would be expected to avoid accumulation of photodynamic chlorophyll intermediate metabolism products that are known to lead to oxidative damage upon exposure of the tissue to light (Hu et al., 1998; Mock et al., 1999; Molina et al., 1999; Ishikawa et al., 2001; Mach et al., 2001; Pružinská et al., 2003; Harpaz-Saad et al., 2007). The enzymatic breakdown of chlorophyll...
into phytol and the primary cleavage product of the porphyrin ring occurs in four consecutive steps catalyzed by chlorophyllase (Chlase), recently shown to catalyze the rate-limiting step in photosynthetic leaves (Harpaz-Saad et al., 2007), magnesium-dechelatase (not yet demonstrated to be an enzymatic step), pheophorbid a oxygenase (PaO), and red chlorophyll catabolite (RCC) reductase, respectively (Chlase: Jacob-Wilk et al., 1999; Tsuchiya et al., 1999; PaO: Prużynská et al., 2003; Ałós et al., 2006; RCC reductase: Wüthrich et al., 2000; Fig. 1). Further downstream, the porphyrin breakdown products are modified and exported to the vacuole, where they are stored indefinitely (Hörtenersteiner, 1999, 2004; Matile et al., 1999; Takamiya et al., 2000). Beyond the established enzymatic chlorophyll breakdown pathway, recent data from stay-green mutants suggests the involvement of an additional player in chlorophyll degradation: the SGR gene product, which likely functions upstream of the catabolic enzymes (Aubry et al., 2008) and has been suggested to function as a destabilizer of the light-harvesting complex (LHC; Park et al., 2007; Fig. 1).

Citrus are classified as non-climacteric fruit; however, a role for ethylene during ripening, and in particular in chlorophyll breakdown during fruit color-break, is well established (Purvis and Barmore, 1981; Goldschmidt et al., 1993; Porat et al., 1999). Furthermore, the application of exogenous ethylene dramatically enhances the speed of color-break of detached mature green citrus fruit, which is a slow process for fruit left untreated on trees (Amir-Shapira et al., 1987; Iglesias et al., 2001). Thus, induction of fruit color-break by exogenous ethylene provides a defined time frame that enables the study of chlorophyll breakdown in citrus fruit at the molecular level. Indeed, previous work has demonstrated the correlation between chlorophyll breakdown and expression of the gene encoding Chlase in ethylene-treated fruit (Jacob-Wilk et al., 1999), suggesting that regulation of chlorophyll catabolism at the gene expression level occurs. However, Chlase gene expression is not increased during color-break of untreated fruits (Jacob-Wilk et al., 1999), raising the possibility that an additional posttranslational mode of regulation may be involved in regulating its function (Harpaz-Saad et al., 2007).

Intracellular localization is often one of the keys to understanding enzyme function and regulation in vivo and has been a controversial issue with regard to the enzyme Chlase. Krossing (1940) was first in demonstrating Chlase activity in a chloroplast fraction, followed by others (barley [Hordeum vulgare] leaves: Matile et al., 1997; citrus fruit: Trebitsh et al., 1993; citrus leaves: Garcia and Galindo, 1991; Ginkgo (Ginkgo biloba) leaves: Okazawa et al., 2006), some of which associated Chlase activity specifically with the plastid membrane fraction (Ardao and Vennesland, 1960; Terpstra, 1974, 1976; Tarasenko et al., 1986; Harpaz-Saad et al., 2007) or specifically with thylakoids (Okazawa et al., 2006). However, different attempts to establish the association of Chlase with specific thylakoid-located pigment-protein complexes have yielded ambiguous results. On the one hand, Chlase activity has been detected in components of the LHC of PSII (LHC II) that were separated electrophoretically (Tarasenko et al., 1986; Brandis et al., 1996), while on the other hand, attempts to separate solubilized complexes by density gradient centrifugation have failed to confirm an intimate association between Chlase and LHC II (Schellenberg and Matile, 1995; Brandis et al., 1996). Moreover, purified LHC II has been found to be devoid of Chlase activity (Schellenberg and Matile, 1995). Hirschfeld and Goldschmidt (1983) showed that Chlase activity is present in the thylakoid-free plastom plastoplasm membranes prepared from citrus fruit, suggesting that Chlase is present in plastid envelope membranes (the only membranes present in chloroplasts). More recent work (Matile et al., 1997) demonstrated association of Chlase activity with the plastid inner-envelope membrane from barley leaves, similar to the results obtained for PaO, the third enzyme of the chlorophyll catabolic pathway (Matile and Schellenberg, 1996).

The relatively recent cloning of genes encoding Chlase from several species has added to the confusion. Citrus and Chenopodium Chlase were suggested to be N-terminally processed based on comparison of the gene open reading frame to the N-terminal sequence of the purified protein. However, the apparently cleaved N-terminal sequences are not typical of N-terminal plastid transit peptides (Jacob-Wilk et al., 1999; Tsuchiya et al., 1999), leading to speculation that some Chlases may be targeted elsewhere in the cell (Takamiya et al., 2000). Recent work, showing that expression of the two known Arabidopsis (Arabidopsis thaliana) Chlase genes (AtCLH1 and AtCLH2) fused to GFP (C and N terminally) yielded cytoplasmic-localized proteins, questions the actual role of the known Chlase genes/enzymes in chlorophyll catabolism (Schenk et al., 2007).

In this work, we studied the dynamics of citrus Chlase in chlorophyll breakdown within its natural fruit flavedo tissue during ethylene-induced color break. We demonstrate, using in situ immunofluorescence, that Chlase is localized to plastids, and mostly associated with chlorophyll, in fruit flavedo. Levels of Chlase enzyme in the flavedo cells/plastids are negatively correlated with chlorophyll levels, further supporting the role of citrus Chlase in chlorophyll breakdown. Immunoblot and protein-mass spectrometry (MS) analyses demonstrate that Chlase initially accumulates as an approximately 35-kD precursor, which is subsequently N-terminally processed to approximately 33-kD mature forms by cleavage at one of three consecutive amino acids.

RESULTS

In Situ Immunofluorescence Establishes Localization of Chlase to the Plastid in Ethylen-Treated Citrus Fruit Peel

The intracellular location of Chlase has been subject to continuous controversy. We therefore pursued a
study on the intracellular localization of citrus Chlase within the endogenous lemon (Citrus limon) fruit peel tissue during ethylene-induced fruit color-break. In situ immunofluorescence was chosen as the method to study localization in the endogenous citrus tissue, because it provides an unbiased picture of the distribution of any given protein within the cell. Mature green lemon fruits were treated with ethylene for 24 h to induce chlorophyll breakdown as well as Chlase expression. Flavedo (the outer pigmented part of the peel) of the ethylene-treated lemons was sectioned, fixed, and subjected to immunofluorescence analysis. To obtain fixed scanning conditions, the confocal parameters were calibrated on control cross-section samples. Emission levels, green (515–525 nm) and red (660 nm), were fixed and used on all samples. Confocal microscopy images were recorded for brightfield, green fluorescence of the Alexa-488 secondary antibody and red autofluorescence of chlorophyll. Control sample sections dressed only with the secondary antibody (Alexa-488-conjugated anti-rabbit) do not show any fluorescence of the Alexa-488 fluorophore in any of the cells/chloroplasts (representative cell shown in Fig. 2A), while chlorophyll fluorescence of the chloroplasts is clearly visible (Fig. 2A, 2 and 3). Sections dressed with affinity-purified anti-citrus Chlase antibody followed by the secondary antibody showed fluorescence of the Alexa-488 fluorophore, corresponding to Chlase detection, in an estimated 50% of the flavedo cells. The Alexa-488 fluorescence (green) was limited almost exclusively to plastids (Fig. 2B, 1), overlapping with the red fluorescence of chlorophyll (Fig. 2B, 2 and 4). Low levels of fluorescence were also detected in small spherical structures distributed throughout the cells (Fig. 2B, 1). The intra-organellar localization of Chlase has been no less controversial than the intracellular location, and data supporting plastid localization mostly point to localization in the inner envelope membrane of the plastid (Hirschfeld and Goldschmidt, 1983; Matile et al., 1997). However, these data specifically addressed potential inner envelope localization and...
were not designed to provide an unbiased picture of the intra-organellar distribution of Chlase. We therefore induced color-break in citrus fruit by ethylene and looked at magnifications of flavedo cell plastids in which Chlase was detected by in situ immunofluorescence. Although the confocal microscope resolution is limiting for analysis of intra-organellar compartmentalization, the high magnifications show colocalization of most of the Alexa-488 and the chlorophyll fluorescence within the plastid, suggesting that most of the Chlase protein is associated with the photosynthetic membranes. This observation was supported by the confocal microscope colocalization program analysis tool, where the red channel, corresponding to the emission of chlorophyll autofluorescence (Fig. 2C, 1), and the green channel, corresponding to Chlase immunofluorescence (Fig. 2C, 2), were merged, resulting in a colocalization signal shown in yellow (Fig. 2C, 3). The percentage of the yellow signal from total fluorescence observed was calculated to be approximately 74.6% (SD = 1.5; an average of three different images).

Expression of Chlase Is Correlated with Chlorophyll Breakdown at the Cellular Level

It was previously shown that treatment of maturing citrus fruit with exogenous ethylene dramatically enhances the speed of color break at the whole tissue level (Shimokawa et al., 1978; Purvis and Barmore, 1981). The current work, based on confocal microscopy, shows that ethylene-induced chlorophyll breakdown is not synchronous between cells of the flavedo tissue; while some cells display reduced chlorophyll levels after 24 h of ethylene treatment, in other cells the chlorophyll appears to be still intact (Fig. 3). Thus, it appears that the ethylene response kinetics is not uniform in citrus flavedo tissue at the cellular level. We therefore investigated the correlation between ethylene-induced chlorophyll breakdown and Chlase expression at the cellular level by in situ immunofluorescence (Fig. 3), monitored by laser scanning confocal microscopy. Immunofluorescence of two representative sample sections is presented (Fig. 3), showing a clear negative correlation between Chlase protein levels (green fluorescence; Fig. 3, A and B, 1) and chlorophyll levels (red fluorescence; Fig. 3, A and B, 2) also presented in a merged format (Fig. 3, A and B, 3). Chloroplasts in cells displaying high levels of Chlase appear to have undergone massive chlorosis.
while chloroplasts in cells lacking Chlase appear to contain mostly intact chlorophyll. We note that quenching of the chlorophyll fluorescence by the Alexa-488 fluorophore or vice versa has been ruled out (Alexa-488 excitation and emission = 494 and 519 nm, respectively; chlorophyll a/b excitation and emission = 425/460 and 650/670 nm, respectively), and thus the results reflect the actual levels of fluorescence of chlorophyll and Alexa-488.

Accumulation and Processing Kinetics of Chlase Protein in Response to Ethylene Induction

Purified citrus Chlase protein was previously shown to be de novo synthesized following ethylene treatment and was characterized, based on N-terminal sequencing, to be a processed protein lacking the N-terminal 21 amino acids encoded by the cDNA (Trebitsh et al., 1993; Jacob-Wilk et al., 1999). However, the precursor form of citrus Chlase, as well as putative precursors of Chlases from other plant species, were never observed in their respective endogenous plant systems, and it is unclear whether the precursor forms accumulate or are immediately processed (Trebitsh et al., 1993; Tsuchiya et al., 1999). To establish the kinetics of citrus Chlase protein expression and processing in response to ethylene during fruit color break, mature green lemon fruits were treated with (20 μL L⁻¹) ethylene for up to 120 h. The flavedo tissue was harvested, and proteins were extracted and analyzed by immunoblot using citrus Chlase-specific antibodies (Fig. 4). Chlase protein was not detected in nontreated fruit flavedo (time point 0) but accumulated after 12 h of ethylene treatment (Fig. 4A), predominantly in the “precursor” form (approximately 35 kD). Levels of the precursor form were maintained for 24 h, after which they gradually decreased concomitantly with a dramatic increase in the levels of the “mature” Chlase form (approximately 33 kD). Fruit color-break was monitored and documented at the same time points used for protein extraction (Fig. 4B). Deep green mature lemons (0 h) became light green after the first 24 h, yellow-green after 48 h, and bright yellow after 120 h, demonstrating the correlation between Chlase protein accumulation, processing (maturation), and fruit peel color-break.

Identification of Precursor and Mature Citrus Chlase Forms by Mass Spectrometry

The kinetics of citrus Chlase precursor expression and processing during fruit color-break presented above was inferred based on immunodetection using anti-citrus Chlase antibodies; however, further verification of Chlase forms and the putative processing site are required to reach established conclusions. Therefore, the putative precursor and mature Chlase forms were purified from ethylene-treated lemon peel (sample A after 12 h of treatment and sample B after 120 h of treatment) by immunoprecipitation followed by SDS-PAGE. Purified bands of approximately 35 kD and approximately 33 kD were subjected to proteolysis followed by identification of peptides by tandem MS (MS/MS) analysis.

Both protein bands yielded peptides confirmed by MS/MS to be Chlase versions. MS analysis of peptides derived from the approximately 35-kD protein band resulted in 44% overall sequence coverage corresponding to citrus Chlase (data not shown). The tryptic polypeptide (K)PAASVQGTPLATLPLPVTR, which starts at amino acid 9 of the Chlase open reading frame, was identified in the sample (Fig. 5), confirming that the approximately 35-kD protein band is the precursor Chlase version that includes the first 21 amino acids encoded by the open reading frame.

MS analysis of peptides derived from the approximately 33-kD protein band resulted in 74% overall sequence coverage corresponding to citrus Chlase (data not shown). To explore possible N-terminal processed peptides, arbitrary in silico sequence frag-
CO2 levels below 1%. The flavedo of the treated and control fruit was once every 24 h, followed by injection of fresh ethylene, to maintain 72, or 120 h in a 600-L sealed container. The container was ventilated and (3) ATATLPVFTR @ m<sub>H</sub> 1,076.614 (Fig. 5). The MS/MS measurement provides daughter ion pattern data that confirms their predicted by the peptide hydrophobicity calculator (Krokhin et al., 2006). The MS/MS measurement provides daughter ion pattern data that confirms their sequence identities (Table I) and demonstrates that the approximately 33-kD protein band contains the processed/mature Chlase versions. As all three peptides appeared in the same spectrum (i.e. they were acquired under identical instrument conditions), rough quantity estimation was performed based on their monoisotopic peak intensities. On this basis, the sample appears to contain approximately 63% ATATLPVFTR (1, i.e. corresponding to processing after amino acid 21), approximately 33% TATLPVFTR (2, i.e. corresponding to processing after amino acid 20), and approximately 4% ATATLPVFTR (3, i.e. corresponding to processing after amino acid 19), demonstrating that Chlase processing occurs not only following amino acid 21 of the precursor, as previously known, but rather at three consecutive positions.

Due to continuous controversy in the literature, determining Chlase enzyme intracellular localization has become a key to understanding its role, mode of action, and regulation in the process of chlorophyll breakdown. In this work, we provide an unbiased localization study demonstrating that citrus Chlase expressed during ethylene-induced fruit color-break is a plastid-localized protein. Furthermore, Chlase protein levels are clearly negatively correlated with chlorophyll levels at the cellular level. Both findings are supportive of the role of Chlase in chlorophyll catabolism in the plastid during citrus fruit color break. This conclusion is further supported by previous work demonstrating that citrus Chlase transiently expressed in tobacco (Nicotiana tabacum) leaf protoplasts is localized to the chloroplast and leads to chlorophyll breakdown in vivo (Harpaz-Saad et al., 2007). We note that our results differ from recent data suggesting cytoplasmic localization of Arabidopsis Chlases based on transient expression of N- and C-terminal GFP fusions (Schenk et al., 2007). Similarly, our previous attempts to reveal the subcellular localization of citrus Chlase by transient expression of both precursor and mature Chlase fused to GFP (N-terminal, C-terminal, and internal fusions) in tobacco protoplasts resulted in cytoplasmic localization comcomitant with the loss of in vivo enzyme function (Harpaz-Saad et al., 2007). We therefore speculate that GFP fusions may interfere with the natural localization of Chlases due to masking of amino- and carboxy-terminal targeting and modification signals (Tian et al., 2004). We also note that the N terminus of most Chlases does not conform to any known criteria for chloroplast transit peptides and recently provided evidence that the citrus Chlase N terminus does not function as a plastid-targeting transit peptide (Harpaz-Saad et al., 2007). Therefore, it is likely that a different domain of Chlase (i.e. not the N terminus) is responsible for plastid targeting, and perhaps it is not surprising that the amino-terminal 47 amino acids of Arabidopsis Chlase (AtCLH2) did not direct a fused GFP reporter to the plastid (Schenk et al., 2007). Based on our current results, generated on the endogenous enzyme in a natural system, we suggest that citrus Chlase and its homologs in other plant species are plastid localized.

Beyond the intracellular plastid localization that was established in this work, Chlase intra-organelar localization is still a major challenge, limiting our understanding of the regulation of Chlase function. Matile et al. (1997) demonstrated enrichment of Chlase activity in purified Brassica and barley inner envelope chloroplast membranes, suggesting that the enzyme is located in the envelope membrane and is spatially separated from the chlorophyll located in the photosynthetic membranes. The interaction between the enzyme and the substrate was speculated to be “latent” and to involve proteins that function in transporting chlorophyll molecules from the photosynthetic membranes to the proposed sites of breakdown in the inner envelope membrane. However, the results of Matile et al. (1997), suggesting inner envelope membrane localization, account for only 6% of the total Chlase activity, while the remaining 94% of Chlase enzyme may possibly be located elsewhere and warrant further investigation. While the resolution of the confocal microscope is insufficient to provide a complete picture
of intra-organellar localization, our results showing colocalization of approximately 75% of the chlorophyll and Chlase signals suggests that the majority of Chlase enzyme is associated with the photosynthetic membranes. This finding is in line with recent results that suggest that Chlase and its substrate chlorophyll come into contact when the enzyme reaches intact chlorophyll located in the photosynthetic membranes (Harpaz-Saad et al., 2007). Yet, some of the Chlase enzyme is clearly located in the plastid envelope membrane both in citrus photosynthetic membrane-free chromoplasts and in barley and Brassica chloroplasts (Hirschfeld and Goldschmidt, 1983; Matile et al., 1997). Because recent results suggest contact between the envelope and photosynthetic membranes (Shimoni et al., 2005), perhaps the mobilization of envelope-located Chlase to the stromal lamellae is facilitated by membrane trafficking.

Accumulation of the Chlase precursor form in its natural tissue was not previously seen in any plant species and was made possible by modulating protein extraction procedures. In this work, we show that during ethylene-induced color-break, citrus Chlase initially accumulates primarily in the precursor form (approximately 35 kD), which is subsequently N-terminally processed to a mature enzyme form (approximately 33 kD), demonstrating that processing is not an immediate event. The role of Chlase N-terminal processing in chlorophyll breakdown during citrus fruit color-break remains unresolved. However, previous work involving expression of citrus Chlase in heterologous systems suggests that the cleaved N-terminal 21 amino acids are not necessary for targeting the protein to the plastid, and their removal results in a more active enzyme in vivo (Harpaz-Saad et al., 2007). Furthermore, detection of both precursor and mature citrus Chlase forms in chloroplasts, purified from tobacco protoplasts transiently expressing citrus Chlase, suggests that processing occurs within the plastid and thus may be involved in enzyme regulation.

The N-terminal processing site of citrus Chlase following amino acid 21 was previously inferred by comparing the N-terminal amino acid sequence of Chlase (starting at Met-1) to the open reading frame encoded by the cDNA (Jacob-Wilk et al., 1999). In the current work, more sensitive tools (MS) were used to show that processing occurs at three consecutive positions: following amino acids 19, 20, and 21 at a ratio of 4%:33%:63%, respectively. The current MS/MS results conform to the previous Edman degradation results.
(Trebitsh et al., 1993), because the most abundant form of mature Chlase (approximately 63%) is the version processed following amino acid 21. The functional significance of multiple processing sites is not clear, but a similar example has previously been described (Zybailov et al., 2008). One possibility is that the processing protease is not precise, resulting in multiple processing sites at different efficiencies. A second possibility is that a stepwise proteolysis is catalyzed by one or more proteolytic enzymes resulting in different lengths of the N terminus of the semi-tryptic peptide detected. Whether or not a stepwise processing has any regulatory significance is currently unclear and needs to be further investigated.

The N-terminal processing site has been experimentally determined up to now for only two Chlases: Citrus sinensis (Trebitsh et al., 1993; Jacob-Wilk et al., 1999; this work) and Chenopodium album (Tsuchiya et al., 1999). While the similarity of the N-terminal sequences between these Chlases is extremely low, processing occurs in both proteins at amino acids of similar nature, mostly before or after Thr in Citrus (this work) and before Ser in Chenopodium (Tsuchiya et al., 1999). It remains to be determined whether N-terminal processing in all plant Chlases occurs at Ser/Thr residues and what other features (i.e. sequence or structural features) direct processing site specificity.

Finally, much of the published data regarding the regulation of the chlorophyll breakdown pathway, and specifically regarding Chlase, was obtained in senescing leaves, involving the chloroplast-gerontoplast developmental transition (Matile et al., 1997; Fang et al., 1998; Kariola et al., 2005; Ben-Yaakov et al., 2006; Schenk et al., 2007) or in response to induction by mediators of biotic stress (Tsuchiya et al., 1999; Benedetti and Arruda, 2002). Here, we investigated the role of Chlase in fruit color-break, reflecting the developmentally regulated transition of chloroplasts to chromoplasts. We note that chromoplasts differ markedly from gerontoplasts, which are the plastid structures of senescing tissue (Sitte et al., 1980; Bouvier and Camara, 2007; Krupinska, 2007). Indeed, the transition to chromoplasts involves both catabolic and anabolic processes, while the transition to gerontoplasts during senescence is strictly catabolic. In this context, we note that recent work questions the role of Arabidopsis Chlases (AtCLH1 and AtCLH2) in chlorophyll breakdown during senescence, because double mutant plants do not display a significant delay in senescence-associated chlorophyll breakdown (Schenk et al., 2007). In contrast, data regarding the Chlase of citrus fruit in the native and the heterologous systems suggests that it plays a major role in chlorophyll catabolism in vital tissue (Harpaz-Saad et al., 2007; this work). We further note that AICLH1 was demonstrated to be involved in chlorophyll turnover in healthy plants or in breakdown following treatment with methyl-jasmonate or in response to high light (Kariola et al., 2005). These

Table 1. Confirmation of N-terminal semi-tryptic peptides by MS/MS

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results demonstrate a role for AtCLH1 in chlorophyll breakdown under natural physiological conditions in vital tissues, such as during natural turnover, in response to wounding or photobleaching. Thus, it is possible that regulation of chlorophyll breakdown may differ between vital physiological developmental stages (i.e. photosynthetic leaves and ripening fruit) versus senescence, which is a metabolite salvaging step in aging tissue in which diverse catabolic machines are induced. Further work will be needed to investigate whether chlorophyll breakdown during these distinct plastid transitions is indeed regulated differently.

MATERIALS AND METHODS

Plant Material and Treatment

Mature green lemon (Citrus limon) fruit were treated with ethylene (20 μL L⁻¹) at 25°C in the dark for 0, 12, 24, 48, 72, or 120 h in a 600-L (for protein extraction procedures) or 4-L (for immuno in situ procedure) sealed container. The container was ventilated once every 24 h or 12 h, respectively, followed by injection of fresh ethylene to maintain CO₂ levels below 1%.

Extraction and Analysis of Protein from Citrus Flavedo

Flavedo Harvesting

The flavedo (the outer, pigmented part of the fruit peel) was peeled, frozen in liquid nitrogen, and ground to a powder. Ground powder samples were acetone precipitated by addition of 5 volumes (w/v) of cold acetone (−20°C), followed by overnight incubation at −20°C. Samples were then centrifuged for 10 min at 15,000g, 4°C.

Protein Extraction

Acetone-precipitated protein samples (45 mg) were homogenized and incubated in 450 μL of 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 15,000g at 25°C, the supernatant was transferred to a new tube, and the centrifugation step was repeated two additional times 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.

Western-Blot Analysis

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes (Schleicher and Schuell) as described (Page and Thorpe, 1996). Membranes were blocked and incubated overnight with the following antibodies: mouse anti-citrus Chlase polyclonal antibodies diluted 1:1,000 (Harpaz-Saad et al., 2007); and rabbit anti-LHC II polyclonal antibodies diluted 1:40,000 (Agrisera). Immunoblots were developed using the SuperSignal West Dura substrate (Pierce) after incubation for 1 h with goat anti-mouse horseradish peroxidase-conjugated secondary antibody diluted 1:10,000 (Jackson ImmunoResearch Laboratories). Dressed immunoblots were visualized with horseradish peroxidase-conjugated secondary antibody diluted 1:10,000 (Jackson ImmunoResearch Laboratories) in PBS containing 0.05% Triton X-100 and 1% BSA without antibody.

From this stage on, all procedures were carried out in the dark. Samples were incubated with the secondary antibody (Alexa-488-conjugated goat anti-rabbit; Jackson ImmunoResearch Laboratories) in PBS containing 0.05% Triton X-100 and 1% BSA for 1.5 h at 25°C, followed by three 30-min washes with PBS. The tissue samples were then mounted with Elvanol (DuPont) under a coverslip.

Confocal Microscopy and Image Analysis

Images were acquired using a DXI fully automated Olympus microscope equipped with a 488-nm argon laser for Alexa-488 excitation and a narrow-band emission filter (515–525 nm) to filter Alexa-488 from chlorophyll green autofluorescence, a BA 660 IF emission filter for chlorophyll red autofluorescence, and a PLAPO60XWLSM (numerical aperture 1.00) objective. Transmitted light images were acquired using Nomarski differential interference contrast. Image analysis was carried out using the flow view software (Olympus).

Antibody Affinity Purification

Polyclonal anti-citrus Chlase antibodies were raised in rabbit (immunological services, Weizmann Institute of Science, Rehovot, Israel) against a recombinant StrepTag-thioredoxin-Chlase/N fusion protein expressed in bacteria as previously described (Harpaz-Saad et al., 2007). Anti-Chlase antibody affinity purification was performed by affinity binding to recombinant Chlase protein cross-linked to cyanojen bromide-activated Sepharose 4B as described by the manufacturer (GE Healthcare Bio-Sciences AB). Eluted antibody concentration was determined using the BCA Protein Assay kit as described by the manufacturer (Pierce).

Immunoprecipitation and Gel Purification of Chlase Versions

Acetone powder samples (3 g) of ethylene-treated (12 and 120 h) lemon fruit peel were homogenized and incubated in 30 mL of radioimmunoprecipitation assay (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 15,000g at 4°C, the supernatant was transferred to a new tube, and the centrifugation step was repeated two additional times 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 to 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 complexes was collected by centrifugation at 40g, 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 420g, 20°C. The protein containing supernatant was transferred to a new tube.

To separate and isolate Chlase protein versions, immunoprecipitated protein samples were separated by 12.5% 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). Gels were then transferred to fresh fixation solution containing 10% [w/v] ammonium sulfamate; 10% phosphoric acid; 0.12% colloidal Coomassie Brilliant Blue G-250 [Fluka]; 20% methanol, in HPLC grade distilled, deionized water. Gels were stained for 16 to 20 h, washed twice for 20 min in destaining solution (20% ethanol), followed by washing with distilled, deionized water for 20 min. Protein gel bands of the Chlase precursor and mature versions were carefully excised and submitted to analysis by MS as detailed below.
Mass Spectrometric Analyses of Protein Bands

Protein gel slices was first subjected in situ to a full tryptic digest overnight. In preliminary examinations, the digest was 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., 2003). For more detailed analyses, the proteolyzed fragments were separated into 40 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). We note that some protein samples were identified as a service in the Smoler Proteomics Center in Israel.

To explore possible N-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 10 ppm variation in mass and ±5 min in retention time.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF160869.

ACKNOWLEDGMENTS

We thank Ms. Hanita Zemach for advice and assistance in preparing samples for in situ immunofluorescence and Dr. Einat Sadot for generous contributions of antibodies. We thank Dr. Hiranit Koltsi for use of the Vibratome for tissue sectioning, and Dr. Edna Pesis, Dr. Amnon Lichter, Dr. Ammon Lers, Mr. Oleg Feygenberg, and Mr. Yohanani Zutahy for advice and assistance with ethylene treatments. We thank Arten Hadarim (R-10 Agriculture, Nir Zvi, Israel) for generous contributions of lemons.

Received June 17, 2008; accepted July 10, 2008; published July 16, 2008.

LITERATURE CITED


Hö rtensteiner S (2004) The loss of green color during chlorophyll degrada-


Iglesias DJ, Tadeo FR, Legaz F, Primo-Millo E, Talon M (2001) In vitro sucrose stimulation of colour change in citrus fruits epicarps: interac-
tions between nutritional and hormonal signals. Plant Physiol 112: 244–250


Jacob-Wilk D, Holland D, Goldschmidt EE, Rivov J, Eyal Y (1999) Chlo-
rophyll breakdown by chlorophyllase: isolation and functional expres-
sion of the Chlase1 gene from ethylene-treated citrus fruit and its regulation during development. Plant J 20: 653–661


Krossing G (1940) Versuche zur Lokalisation einiger Fermente in den verschiedenen Zellbestandteilen der Spinatblätter. Biochem Z 305: 359–373


Mach JM, Castillo AR, Hoogstraten R, Greenberg JT (2001) The Arabi-


conferring increased resistance to tobacco mosaic virus. J Biol Chem 274: 4231–4238


