Pathway of Chlorophyll Breakdown in Citrus Fruit Peel, as Compared with Arabidopsis Leaves and Other Plant Systems

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Abstract

The pigment changes of citrus fruit peel [='colorbreak'] are the best visual markers of citrus fruit maturation. Pigment changes of most citrus cultivars consist of breakdown of chlorophyll and buildup of carotenoids, both of which are enhanced by ethylene and delayed by gibberellins. The degreening of citrus fruit by ethylene has served for more than 30 years as a model system for the study of chlorophyll catabolism. The abrupt upsurge of chlorophyllase enzyme activity in response to ethylene prompted further, molecular investigations. Citrus *Chlase 1* overexpression was shown to initiate chlorophyll catabolism in several heterologous systems, supporting the notion that chlorophyllase is a rate limiting enzyme. Confocal microscopy localization has shown that both forms of chlorophyllase [the longer precursor and the shorter, mature form] reside in citrus' plastids. In conclusion, the strong correlation between ethylene induction of citrus fruit colorbreak and chlorophyllase gene expression and protein maturation indicate that chlorophyllase initiates the chlorophyll catabolic pathway in this system.

In Arabidopsis (*Arabidopsis thaliana*), on the other hand, recent work based on analysis of mutants questions the role of chlorophyllase in chlorophyll breakdown during senescence. Moreover, the Hortensteiner group identified an alternative enzyme from Arabidopsis leaves involved in dephytilation, pheophytin pheophorbide hydrolase (PPH), which exhibits specificity to the Mg-free form of chlorophyll (phein), and must, therefore, be preceded by a Mg removal step. This finding raises doubts regarding the role of chlorophyllase as the universal initiator of chlorophyll catabolism and points to the existence of two, alternative catabolic pathways. Examination of the actual roles of chlorophyllase and PPH in various plant systems is presently underway. The hypothesis that ripening fruits and senescing leaves represent distinct modes of chlorophyll catabolism deserves consideration.

INTRODUCTION

The pigment changes of citrus fruit peel, generally known as 'colorbreak', are the most conspicuous external symptoms of citrus fruit maturation. In various early varieties (e.g. Clementine, *Citrus clementina*) the acquisition of internal maturity precedes the external color change; such green fruit get low prices in the fresh citrus fruit market. Gassing with ethylene has been used since the Nineteen Twenties, and is still being used, to enhance the degreening of citrus fruit for commercial purposes. Later on, while ethylene was recognized as the universal 'ripening hormone', gibberellins and cytokinins were found to be effective in delaying senescence and antagonizing the effect of ethylene. Thus, gibberellin-treated citrus fruit proved to be difficult-to-degreen with ethylene. 'Valencia' orange (*Citrus sinensis*) fruit retained on the trees for the next summer were found to reacquire greenish appearance [=regreening]; gibberellins amplified the regreening phenomenon (Coggins and Lewis, 1962). Biochemically, the colorbreak consists of two complementary processes, the breakdown of chlorophyll and the buildup of carotenoids. From the structural viewpoint, the ripening-associated pigment changes reflect the transformation of the photosynthetic chloroplasts into chromoplasts, a process

which was followed closely in various fruits. Temperature, light and mineral nutrition were also shown to affect the natural colorbreak, as well as the regreening of citrus fruit (cf. Goldschmidt, 1988). Still, little is known about the mechanisms underlying the colorbreak processes and, in particular, their enhancement by ethylene.

THE BEGINNINGS: CHLOROPHYLLASE ACTIVITY

The present article focuses on the chlorophyll degradation aspect of citrus' colorbreak phenomena. As late as 1987 the degradation of chlorophyll was regarded as a 'biological enigma' (Hendry et al., 1987). Chlorophyllase has been known since the pioneering work of Willstatter and Stoll (1913) but its role in chlorophyll catabolism was controversial. Nevertheless, the early reports on induction of chlorophyllase activity in citrus peel by ethylene (Barmore, 1975; Shimokawa et al., 1978; Hirschfeld and Goldschmidt, 1983) served as the initial foothold for further, more advanced research. Identification of chlorophyllide a, the immediate product of chlorophyllase activity, in ethylene-treated citrus fruit peel as well as in autumnal, senescing leaves of China tree (*Melia azedarach*) lent further strength to the hypothesis that chlorophyllase is involved in the initial steps of chlorophyll degradation, at least in certain plant senescence systems (Amir-Shapira et al., 1987).

DE-NOVO SYNTHESIS AND GENE EXPRESSION

Ethylene-treated 'Valencia' orange peel proved to be a rich source for isolation and purification of the chlorophyllase protein; the N-terminal sequence of the protein was determined. Antibodies raised against the purified protein were used in immunoblot analyses of crude citrus peel extracts; a strong signal of the chlorophyllase protein was detected in ethylene-treated fruit, while only a trace level of the protein could be detected in control fruit, held in air. Gibberellin and cytokinin treatments partly counteracted the ethylene-induced upsurge in chlorophyllase protein. Thus, ethylene appears to enhance the degreening of citrus fruit through de novo synthesis of the chlorophyllase protein (Trebitsh et al., 1993).

Isolation of the gene encoding citrus chlorophyllase [*Chlase1*] was the next, obvious step (Jacob-Wilk et al., 1999). *Chlase 1* encodes a protein of 329 amino acids, including a sequence domain characteristic of serine-lipases. The *Chlase 1* gene encodes an active chlorophyllase enzyme, as shown by in vitro recombinant enzyme assays. Chlorophyllase expression in 'Valencia' orange peel was found to be low and constitutive during natural fruit development without significant increase towards colorbreak. However, ethylene treatment induced a significant increase in chlorophyllase transcript throughout fruit development, most prominently during the autumnal, natural colorbreak period (Jacob-Wilk et al., 1999).

The lack of detectable increase in chlorophyllase gene expression during the natural colorbreak period [although a slight increase was recently found in 'Comune' clementine (Distefano et al., 2009)], as shown previously for in vitro chlorophyllase enzyme activity (Trebitsh, 1993), may raise doubts with regard to the role of chlorophyllase in the natural breakdown of chlorophyll during citrus fruit ripening. Subsequent work indicated however, that subtle, posttranslational modification of the chlorophyllase protein in planta might be responsible for activation of the chlorophyllase system during the natural breakdown of chlorophyll in ripening citrus fruit.

PRECURSOR AND MATURE FORMS OF CHLOROPHYLLASE

The first gene encoding chlorophyllase that was isolated from citrus based on protein sequence data (Jacob-Wilk et al., 1999) revealed an N-terminally encoded sequence absent from the mature protein. A similar situation was found with the chlorophyllase gene isolated about the same time from *Chenopodium* (Tsuchiya et al., 1999). The N-terminal sequence is short (in citrus, 21 amino acids) and not characteristic of chloroplast transit peptides.

In order to analyze *Chlase 1* gene products in planta we used a ZYMV-based viral

vector infective clone system (AGII) designed for the expression of genes of interest in cucurbits [for details see Harpaz-Saad et al. (2007)]. Two versions of the citrus *Chlase1* gene were cloned into the AGII viral system : (1) full length *Chlase1* consisting of the entire coding sequence of the cDNA(AGII-Chlase); and (2) a *Chlase 1* version corresponding to the mature protein that lacks the first 21 amino acids encoded by the cDNA (AGII-ChlaseΔN). Squash (*Cucurbita pepo*) plants were inoculated with infectious AGII-Chlase and AGII-ChlaseΔN clones, and AGII Chlase-less controls. Plants infected with the empty control viral vector (AGII) and with the vector encoding full length, AGII-Chlase, displayed a normal, dark green phenotype, with minor symptoms of viral infection. By contrast, plants infected with the vector encoding the shorter, mature protein (AGII-ChlaseΔN) developed a distinct, blotchy, chlorotic phenotype, indicating massive chlorophyll degradation. Despite the different phenotypes observed, in vitro assays in crude extracts from plants expressing both forms of *Chlase 1* showed high chlorophyllase activity; the activity in plants infected with the AGII-Chlase was actually ~25% higher than in plants infected with the AGII-ChlaseΔN, which revealed massive breakdown of chlorophyll in planta (Harpaz-Saad et al., 2007). Thus, the in vitro measured chlorophyllase activity may not truly reflect the in vivo situation; lack of apparent increase in chlorophyllase activity during breakdown of chlorophyll (Trebitsh, 1993; Ben-Yaakov et al., 2006) may, in fact, conceal posttranslational processing events that set the in vivo chlorophyllase activity into motion.

In complementary experiments, we analyzed the effect of transient expression of the same *Chlase 1* versions (full length and ChlaseΔN) in tobacco (*Nicotiana tabacum*) protoplasts. Two plasmid constructs were designed to contain the gene encoding green fluorescent protein (GFP) driven by a 35S promoter and either full length Chlase (p35S-Chlase+35S-GFP) or ChlaseΔN (p35S-ChlaseDN+35S-GFP) separately driven by an additional 35S promoter. An additional control construct consisted of the GFP encoding gene driven by a 35S promoter (p35S-GFP). The three constructs were electroporated into tobacco protoplasts for transient expression, and cell fluorescence was visualized by confocal microscopy. Transformed cells were detected by the fluorescence of GFP and chlorophyll levels of those cells were monitored by chlorophyll red fluorescence, which was quantified as chlorophyll area intensity (CAI). Visual inspection revealed partial disappearance of the chlorophyll in the 'full length' expressing protoplasts but only traces of chlorophyll in the ChlaseΔN expressing ones. CAI data indicated 78.2% chlorophyll degradation in the 'full length' expressing protoplasts as against 95.4% degradation in the ChlaseΔN expressing protoplasts.

The results obtained from overexpression of the two versions of *Chlase 1* in both heterologous systems (squash and tobacco) are consistent, and support the notion that the 'full length', precursor chlorophyllase protein is tightly regulated and must undergo some posttranslational changes in order to achieve full enzymatic competence.

LOCALIZATION AND PROCESSING OF CHLOROPHYLLASE IN THE CITRUS SYSTEM

Having shown that *Chlase 1* induces breakdown of chlorophyll in heterologous systems, we decided to get back to the citrus colorbreak system and address the issues of intracellular localization of chlorophyllase and its processing. Using in situ immunofluorescence (Azoulay-Shemer et al., 2008) on ethylene-treated lemon (*Citrus limon*) fruit peel, it was shown that chloropyllase resides exclusively in plastids. At the intra-organellar level, the chlorophyllase fluorescent signal was found to overlap mostly with chlorophyll auto-fluorescence, suggesting association of most of the chlorophyllase protein with the photosynthetic membranes. Confocal microscopy analysis showed that for individual cells the quantity of chlorophyll was negatively correlated with plastid chlorophyllase levels; indicating destruction of the chlorophyll initiated by chlorophyllase. Immunoblot analysis of chlorophyllase in lemon peel during the course of ethylene-induced colorbreak (120h) demonstrated the gradual shift from the 'full length', precursor form of 35 kDa to the mature, 33 kDa form (Fig. 1).

RECENT DEVELOPMENTS IN THE CHLOROPHYLL CATABOLISM AREA

While it is clear from the foregoing that chlorophyllase plays a central role in the ethylene-induced breakdown of chlorophyll in citrus peel, the role of chlorophyllase in leaf senescence has been questioned, since a double mutant of Arabidopsis (*Arabidopsis thaliana*) chlorophyllases (*AtCLH1* and *AtCLH2*) did not display a significant delay of senescence-associated chlorophyll breakdown (Schenk et al., 2007). Further more, a new enzyme, pheophytin pheophorbide hydrolase (PPH) (Fig. 2) was recently suggested to be the major dephytilation enzyme during Arabidopsis senescence, since a loss-of-function PPH mutant was shown to display a stay-green phenotype (Schelbert et al., 2009). However, PPH does not appear to be an initiator of chlorophyll breakdown, since when overexpressed (in the PPH mutant background) it led to chlorophyll breakdown only in senescing leaves, suggesting that a factor further upstream regulates chlorophyll breakdown during senescence. One strong candidate for regulating breakdown of the photosynthetic membranes during senescence is the protein SGR (Fig. 2), which was shown to be mutated in stay-green mutants of a variety of plant species (Hortensteiner, 2009). A stay-green mutant of *Citrus sinensis* has also been recently characterized (Alos et al., 2008). The precise function of SGR is still unknown, however, its association with the light-harvesting chlorophyll binding protein (LHCP) inspired the hypothesis that it functions as a de-stabilizer of the light harvesting complex (Park et al., 2007). Further support for this hypothesis was obtained from transient overexpression experiments of SGR in tobacco leaves, which resulted in dramatic chlorosis.

In contrast to PPH, chlorophyllase was shown to be an initiator of chlorophyll breakdown, since overexpression of the enzyme in healthy young leaves resulted in massive chlorophyll breakdown (Harpaz-Saad et al., 2007; unpublished data). The relationship between Chlase and SGR, if any, and the precise division of labor between the dephytilation enzymes Chlase and PPH under different physiological conditions leading to chlorophyll breakdown, remains to be determined.

It is difficult or rather impossible, to state with certainty at this point which of the suggested catabolic pathways is dominant. Differences between species and organs (e.g. senescing leaves vs. ripening fruits) must be seriously considered. New experimental evidence is desperately needed to in order to resolve this exciting, still somewhat enigmatic issue.

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Figures

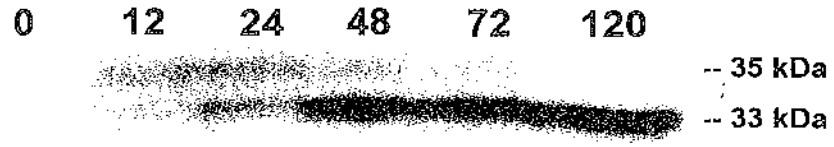

Fig. 1. Expression and processing kinetics of citrus chlorophyllase during ethylene-induced fruit colorbreak. Mature green lemon fruits were treated with ethylene (20 µl L^{-1}) at 25°C in the dark, for 0, 12, 24, 48, 72, 120 h in a 60-liter sealed container. The container was ventilated once every 24 h, followed by injection of fresh ethylene, in order to maintain CO_2 levels below 1%. The flavedo of the treated and control fruit was peeled, frozen in liquid nitrogen and ground to a powder. Total protein from each time point was acetone precipitated, extracted in USB protein extraction buffer, separated by SDS-PAGE (30 µg protein per-lane), blotted, and dressed with anti citrus chlorophyllase antibodies. Protein bands bound by the chlorophyllase-specific antibodies were visualized by chemiluminescence. Relative molecular weights of the bands detected by the antibody are denoted.

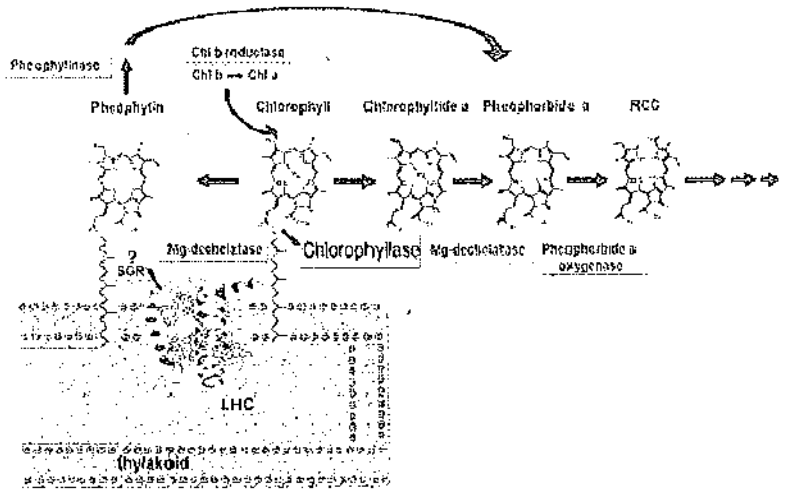

Fig. 2. Chlorophyll Catabolic Pathways. Two theoretical Chlorophyll break-down pathways are outlined: A. Vital tissue/Fruit ripening/Stress pathway, catalyzed by the key enzyme Chlorophyllase, proceeds in the following order: chlorophyll *b*/chlorophyll *a*/chlorophyllide *a*/pheophorbide *a*/red chlorophyll catabolite (RCC)/nonfluorescent chlorophyll catabolites (NCCs). B. Leaf senescence pathway, catalyzed by pheophytinase (PPH) proceeds in the following order: chlorophyll *b*/chlorophyll *a*/pheophytin *a*/pheophorbide *a*/RCC/NCCs. The enzymes involved are as follows: (1) Chlorophyll *b* reductase involved in maintaining the balance between chlorophyll *a* and *b*; (2) Chlorophyllase catalyzes the cleavage of the hydrophobic thylakoid-anchoring phytol chain from the chlorophyll porphyrin ring; (3) Dechelating of the Mg ion (Mg-dechelatase) from chlorophyll/chlorophyllide, has not yet been shown to be an enzymatic step; (4) Pheophorbide *a* oxygenase (PaO) catalyzes the cleavage of the porphyrin ring, resulting in RCC, which is further metabolized by additional enzymes downstream, exported to the vacuole, nonenzymatically converted to NCCs and stored indefinitely. Additionally, the protein SGR, involved in stay-green mutations, is suggested to function upstream (Park et al., 2007) or downstream (Schelbert et al., 2009) of the chlorophyll catabolic enzymes (Chlorophyllase/ Pheophytinase respectively) by de-stabilizing the light-harvesting complex (LHC).