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Chlorophyllase activity in green and non-green tissues of variegated plants

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Abstract

The main objective of this work was to compare activity of chlorophyllase (chlase) in green and non-green sectors of leaves. Our results show that various plants exhibited different abilities and specificities with regard to chlases. Large variations in chlase activity existed in seven all-green plants, and *Pachira macrocarpa* contained significantly higher chlase activity compared to the other plants. The chlase activity was detected in both non-green and green sectors of the leaves; however, non-green parts had significantly higher chlase activity than did green parts in most of the variegated plants. Most of the chlases in all-green and variegated plants exhibited higher substrate preference toward the chl b than chl a. The activity and localization of chlase in *P. macrocarpa* were higher in chloroplast envelope membrane fractions than thylakoid fractions.

Keywords: Chlorophyll; Chlorophyllase; Chloroplast; Variegated plant

1. Introduction

Chlorophyll (chl) is the most abundant photosynthetic pigment in higher plants, and is bound to chl a/b-binding proteins, cytochrome b6 and early-light-inducible proteins embedded in thylakoid membranes of chloroplasts (Bruno and Wetzel, 2004). Chlorophyllase (chlorophyll-chlide hydrolase, chlase, EC 3.1.1.14) is a hydrophobic enzyme that catalyzes the hydrolysis of chl to chlorophyllide (chlide) and phytol, and is present in the leaves throughout their development and in high levels in some species (Kraulter and Hörtensteiner, 2006; Harpaz-Saad et al., 2007). Chlase was shown to be located in either the envelope or thylakoid membrane of chloroplasts (Okazawa et al., 2006). It is thought that chl degradation proceeds only in thylakoids and inner envelope membranes (Tarasenko et al., 1986); however, Guiamet et al. (1999) reported that numerous large plastoglobuli containing chls and chl protein complexes are extruded into the cytosol through the senescent chloroplast envelope membrane. A different localization can originate from various facts, for instance, multigene family, different importing pathways, different expression patterns, and possible post-translational modifications. Since cloning of the first chlase gene from *Chenopodium album* by Tsuchiya et al. (1999), it is clear that prediction of their localization is uncertain based on the sequence. In particular, in the leaves chloroplast-localization of chlase has been questioned recently (Schenk et al., 2007).

Although much research has focused on the biochemical, physiological and molecular properties of chlase (Takamiya et al., 2000; Kariola et al., 2005), literature indicates that little or no information is available regarding its role in variegated plants. Variegation is found in the leaves, stems, and flowers of plants, and sometimes shows red, yellow, and white sectors. The main characteristics of variegated plants include single thylakoid lamellae, irregular granules, aggregations of plastoglobuli, and the absence of starch. The chlase activity of particular leaf parts differs: highest in bright-green parts which turn green, weaker in green, and still weaker in albino parts (Drazkiewicz, 1994). In fact, chlase is not the only factor in regulating chl levels; the chl biosynthesis pathway and developmental factors are also

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involved and they affect chloroplast differentiation in the different sectors of the leaves (Liu et al., 2010). Many problems pertinent to variegation remain unclear. Whether non-green sectors contain a great amount of chlase has not been proven yet. In this study, we demonstrated that the white or yellow sectors in variegated species contain higher activity of chlase compared to the green parts. Further we investigated the putative location of chlase through direct analysis of isolated envelopes and thylakoids in chloroplasts.

2. Materials and methods

2.1. Plant materials

The seeds of seven all-green representative plants (*Pachira macrocarpa*, *Euphorbia pulcherrima*, *Juniperus chinensis*, *Vinca rosea*, *Syzygium samarangense*, *Schefflera arboricola*, and *Ficus microcarpa* cv. golden leaves) and seven representative variegated plants (*Dieffenbachia* cv. Camilla, *Acalypha hamiltoniana*, *Erythrina variegata*, *F. microcarpa* cv. milky stripe, *S. arboricola* cv. yellow stripe, *Dracaena godseffiana* cv. Florida Beauty, and *Aucuba japonica*) were purchased from a local seed company. The seeds were planted in flats, and placed in a controlled-environment greenhouse maintained at 22/18 °C day/night temperature, 60% relative humidity, and a 14-h photoperiod provided by fluorescent and incandescent light. Single seedlings were transplanted into plastic pots containing a medium consisting of peat moss, loamy soil and sand in a ratio of 2:1:1. Plants were watered every other day, and an optimal amount of compound fertilizer solution ($N–P_2O_5–K_2O$, 20–20–20) was applied once a week. The non-green and green sectors of mature leaves were separated by cutting, and used to prepare acetone powder.

2.2. Preparation of acetone powder

To obtain chlorophyll-free cell powder, an acetone powder method described by McFeeters et al. (1971) with minor modification was conducted in this study. Acetone powder was prepared from fresh green and non-green sectors of the leaves independently. The leaves were frozen in liquid nitrogen and ground to powder with a mortar and pestle, and then homogenized with pre-chilled acetone (−20 °C). After centrifuging the homogenate at 3000 × *g* for 5 min at 4 °C, the pellet was collected. The cold acetone extraction procedures were repeated three times in the same manner to remove all traces of chls and carotenoids. The acetone powder was then dried under nitrogen gas and stored at −20 °C until use.

2.3. Purification of thylakoids and envelopes

Chloroplasts were prepared from fresh leaves of *P. macrocarpa* according to the method of Fitzpatrick and Keegstra (2001). The separation and purification of thylakoid membranes and envelopes of chloroplasts were carried out based on Akita et al. (1997). Purified chloroplasts were diluted in swelling medium containing 10 mM Tricine–NaOH and 4 mM $MgCl_2$ at pH 7.6. The mixture was layered on top of a discontinuous sucrose gradient (0.60 and 0.90–1.20 M, containing 10 mM Tricine–NaOH and 4 mM $MgCl_2$ at pH 7.6), and centrifuged at 336,000 × *g* for 1 h in a Beckman SW60Ti rotor. Solutions at 0.46–0.80 and 0.80–1.05 M were collected as representing purified thylakoids and envelopes, respectively.

2.4. Assay of the chlase activity

To determine chlase activities on chl a and b degradation, 100 mg of acetone powder was homogenized with 5 ml extraction buffer, containing 5 mM potassium phosphate (pH 7.0), 50 mM KCl, and 0.24% Triton X-100 for 1 h at 30 °C. After centrifugation at 15,000 × *g* for 15 m, the supernatant was used for the enzyme assay. The assay of the chlase activity followed a modified method of McFeeters et al. (1971). Briefly, the standard reaction mixture was made of 0.1 ml of supernatant, 0.1 ml of substrate (1 μmol/ml chl a or b dissolved in acetone), and 0.8 ml of reaction buffer (100 mM of sodium phosphate (pH 7.0) and 0.24% Triton X-100). The mixture was incubated for 60 min at 30 °C, and the reaction was stopped with 1.0 ml of 10 mM KOH. Thereafter, 1 ml of the mixture was further mixed with 5 ml of hexane/acetone (3:2, v/v) solvent to eliminate the interference of chl. The product of chlorophyllide a or b in the acetone phase was then determined with a Hitachi U-2000 spectrophotometer (Tokyo, Japan) using an extinction coefficient of 74.9 mM^{-1} cm^{-1} at 667 nm and 47.2 mM^{-1} cm^{-1} at 650 nm for chlides a and b, respectively (Trebitsh et al., 1993). One unit of chl a or b degradation activity was defined as the amount of enzyme needed to catalyze the production of 1 μmol chlide a or b/min.

2.5. Statistical analysis

At least three different plants of the same species were used to calculate average chlase activities. The experiment was performed twice independently for the sampling day and biochemical analyses. Data were then analyzed in a completely randomized design using SAS vers. 8.2 (SAS Institute, Cary, NC, USA). For significance levels, means of chl a, b, and a+b degradation activities of the seven evergreen plants were separated by the least significant difference (LSD) test at $p \leq 0.05$ (Table 1). Means of chlase activities on a, b, and a+b degradation between green and non-green sectors of a leaf in the seven variegated plants were compared using an unpaired *t*-test at a 0.05 probability level (Table 2). Comparisons of the activities of chl a, b, and a+b degradation by chlase between envelope and thylakoid tissues were also made using the *t*-test at a 0.05 probability level (Table 3).

2.6. Antibody preparation and Western blot analysis

The chlase gene named *PmCLH1* (accession no. FJ754215) was cloned from *P. macrocarpa*, and its recombinant protein was produced in *Escherichia coli* using T7 promoter system. The recombinant PmCLH1 was eluted from SDS-PAGE according to the method described by Chuang et al. (1996)

Table 1
Chlase activities on chl a, b, a+b, and the ratio of a/b degradation in seven all-green plants. Values with the same capital letters do not significantly differ ($p \leq 0.05$) among species according to one-way ANOVA. Each value is the mean of three replicate determinations.

Species	Chl degradation (nmol/g min)			
	a	b	a+b	a/b
P. macrocarpa	940.5 A	704.2 A	1644.7 A	1.34
E. pulcherrima	495.9 B	511.0 B	1006.9 B	0.97
S. samarangense	113.0 C	131.8 C	244.7 C	0.86
F. microcarpa cv. *golden leaves*	125.0 C	94.3 D	219.3 C	1.33
V. rosea	68.4 D	99.6 D	167.9 D	0.69
J. chinensis	26.2 E	76.9 E	103.1 E	0.34
S. arboricola	29.9 E	59.8 E	89.7 E	0.50

and used to generate polyclonal antibodies in rabbit. Proteins were separated on 12.5% SDS-PAGE and stained with Coomassie brilliant blue R-250. Proteins separated by SDS-PAGE were transferred to the nitrocellulose membrane and blocked with 3% BSA (Sigma-Aldrich, Saint Louis, USA). The membranes were then hybridized with antibodies including anti-*Pm*CLH1, anti-LHC2B, anti-*At*Tic110, and anti-Toc75 separately. The purity of each chloroplast subfractions was examined by Western blot using antibodies against chloroplast outer (derived from pea Toc75 full-length protein) and inner envelope (derived from *Arabidopsis* Tic110, residues 431–1016) proteins (Tu et al., 2004) and a thylakoid membrane protein (derived from spinach LC2B) separately. After washing, the membrane was probed with anti-rabbit secondary antibody conjugated with alkaline phosphatase, followed by developing a BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitro blue tetrazolium) (Sigma-Aldrich, Saint Louis, USA) signal on membranes.

Table 3
Chlase activities on chl a, b, and a+b, the a/b ratio, and percents of a and b degradation in thylakoid membranes and envelopes purified from chloroplasts of *Pachira macrocarpa*. Comparisons with the same capital letters do not significantly differ ($p \leq 0.05$) between green and non-green tissues, according to the *t*-test.

Preparations	Chl degradation (nmol/min)				Chl degradation activity (%)	
	a	b	a+b	a/b	a	b
Envelope	11,180.2 A	7864.4 A	19,044.6 A	1.42	82%	81%
Thylakoid	2522.2 B	1821.5 B	4343.8 B	1.38	18%	19%

3. Results

3.1. Chlase activities in selected all-green plants

Chl a degradation activities of the leaves in the seven all-green plants ranged from 26.2 nmol/g min (*J. chinensis*) to 940.5 nmol/g min (*P. macrocarpa*) (Table 1), suggesting that large differences in chlase levels exist in these plants. The lowest and highest chl b degradation activities were found in *S. samarangense* (59.8 nmol/g min) and *P. macrocarpa* (704.2 nmol/g min), respectively, which also significantly differed. Meanwhile, the total chl a and b degradation activities of *P. macrocarpa* (1644.7 nmol/g min) were 18-fold greater than that of *S. arboricola* (89.7 nmol/g min). The ratio of chl a and chl b degradation by chlase of *P. macrocarpa* and *F. microcarpa* was $\gg 1.0$, whereas those of the other plants was close to 1.0 or even $\ll 1.0$, suggesting higher chlase activity on chl a degradation in *P. macrocarpa* and *F. microcarpa*. However, no significant difference on the chlase activity toward chl a and chl b was observed among species.

Table 2
Chlase activities on chl a, b, a+b, and the ratio of a/b degradation in green and non-green sectors, and the non-green/green ratio of chlase of the leaves in seven variegated plants. Comparisons with the same capital letters do not significantly differ ($p \leq 0.05$) between green and non-green tissues, according to the *t*-test.

Species	Tissue	Chl degradation (*n*mol/g min)				Non-green/green ratio of chl degradation	
		a	b	a+b	a/b ratio	a	b
F. microcarpa cv. *milky stripe*	Green	109.1 B	118.9 B	228.1 B	0.92	1.58	2.30
	Non-green	172.6 A	273.0 A	445.6 A	0.63		
D. godseffiana cv. *Florida Beauty*	Green	89.0 A	133.7 A	222.7 A	0.67	1.10	1.10
	Non-green	97.5 A	147.0 A	244.4 A	0.66		
Dieffenbachia cv. *Camilla*	Green	57.9 B	86.1 B	144.0 B	0.67	1.42	1.37
	Non-green	82.4 A	118.1 A	200.6 A	0.70		
A. hamiltoniana	Green	265.9 A	269.3 A	535.2 A	0.99	0.63	0.78
	Non-green	167.4 B	211.3 B	378.7 B	0.79		
A. japonica	Green	151.4 A	109.9 B	261.3 A	1.38	0.69	1.69
	Non-green	104.0 B	185.9 A	289.9 A	0.56		
E. variegata	Green	53.7 B	62.8 B	116.5 B	0.86	1.61	1.68
	Non-green	86.3 A	105.7 A	191.9 A	0.82		
S. arboricola cv. *yellow stripe*	Green	69.6 B	80.0 B	149.6 B	0.87	1.62	1.81
	Non-green	113.0 A	145.1A	258.1 A	0.78		

3.2. Chlase activities in green and non-green sectors of variegated plants

Table 2 illustrates comparisons of chlase activities between green and non-green sectors of the leaves of seven variegated plants. In *F. microcarpa*, *Dieffenbachia*, *E. variegata*, and *S. arboricola*, levels of chl a, b, and a+b degradation by chlase in non-green tissues were significantly higher than those in green tissues, suggesting that the chlase activity was enriched in non-green tissues. However, the green parts of *A. hamiltoniana* leaves showed significantly higher activities of chlase on chl a, b, and a+b degradation compared to non-green parts. Interestingly, all chl a/b degradation ratios were <1.0 in both green and non-green sectors of all plants, with the exception of an elevated a/b ratio (1.38) only in the green sector of *A. japonica*. In green sectors of *A. japonica*, the chlase activity on chl a degradation was significantly higher than chl b whereas in non-green sectors was inverted (exhibited significantly higher chlase activity toward chl b). Furthermore, in all variegated plants, the non-green/green ratios of both chl a and b degradation were >1.0, except for *A. hamiltoniana*, suggesting that chlase activities in non-green parts were more active than those in green parts of variegated plants.

3.3. Localization of chlase

P. macrocarpa showed significantly higher chlase activity compared to the other plants (Table 1). Therefore, thylakoid and envelope tissues were separated from chloroplasts of *P. macrocarpa* and purified, and their chl degradation activities were determined and were shown in Table 3. Levels of the chlase activity toward chl a, b, and a+b degradation within the envelope were 4 times higher than those in the thylakoid.

3.4. Western blot analysis of chlase in subcellular fraction of chloroplast

Antibodies of pea Toc75 and *Arabidopsis* Tic110 show minimal cross-reactivity to *P. macrocarpa* chloroplast membrane proteins (data not shown), however, LC2B is relatively enriched in thylakoid fraction (Fig. 1). Chlase gene, *PmCLH1*, from *P. macrocarpa* leaves was cloned, and the recombinant *Pm*CLH1 was used to immunize rabbits for preparing polyclonal antibody as described in Materials and methods. The prepared antibody was used to detect the localization of chlase in subcellular fraction of chloroplast. The results obtained indicate that chlase, at least *Pm*CLH1, resides in the inner envelope of chloroplast but not thylakoid (Fig. 1).

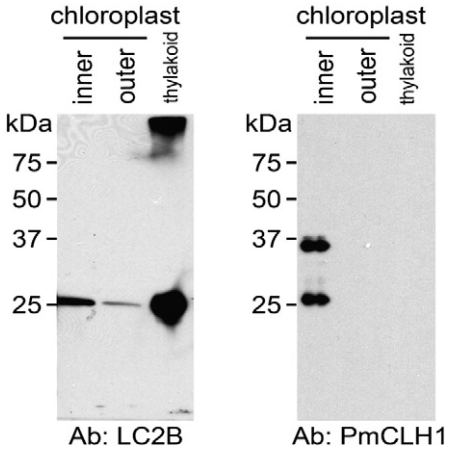

Fig. 1. Western blot analysis of chloroplast inner and outer envelopes, and thylakoid total proteins purified from *P. macrocarpa* leaves. Chloroplast was isolated from *P. macrocarpa* leaves by Percoll gradient centrifugation, and the inner envelope, outer envelope, and thylakoids were further fractioned by discontinuous sucrose gradient. Total proteins (5 μg) were loaded and separately probed by antibodies against LC2B (left panel) and *Pm*CLH1 (right panel).

4. Discussion

Chlase is present in all plants in various quantities throughout their entire life, and the chlase activity has been described in numerous plants and algae species (Hornero and Minquez, 2001). The amount of chlase in plants is affected by many external and internal factors, such as irradiance, temperature, water stress, osmotic and saline–osmotic stresses, heavy metals, fertilization, infections, age, tissues, and growth regulators (Abdel-Basset et al., 1995). The function of chlase is associated with the physiological state of leaf senescence, fruit maturity, and damage control (Azoulay-Shemer et al., 2008). In this study, chlase in different plants exhibited different abilities and specificities on chl a and b degradation. Tables 1 and 2 indicated that the chlase activity was significantly different among the all-green plants but not in variegated plants. Moreover, the higher chlase activity toward chl b but not chl a was detected in most of the cases. All of these findings infer that the functional activity of chlase in all-green plants was tightly regulated when compared to the variegated plants. Chlase is not an essential enzyme involved in senescence-related chl degradation (Liao et al., 2007; Zhou et al., 2007), however, it influences the chl a/b ratio in wild-type and chlase transgenic plants have been reported (Beneditti and Arruda, 2002). In general, the expression level of chlase was negatively correlated with chl a/b ratios in plants. The chl b might be the preferred substrate for chlase in chl b to chl a conversion and/or chl b degradation immediately. Our data support this issue and also provide important information on resource materials for researchers interested in genetic and physiological aspects of the chlase activity in plants.

Ketsa et al. (1999) studied two varieties of mango, one yellow and the other green when ripe, and found different chlase activities between them. Roca and Minquez (2003) illustrated that in olives with high chl contents, chlase remained latent, and oxidative enzymes took part in chl disappearance due to the absence of chlorophyllides. However, in olives with low chl contents, both chlase and oxidative enzymes were responsible for chl degradation. Win et al. (2006) demonstrated that the chlase activity in peel of limes was positively correlated with green to yellow color change and increased during ripening. In our study, during the leaf pigmentation period, chlase displays different activities on chl a and b degradation that may play very different roles in different

parts of the leaf depending on the species of the variegated plant (Table 2). The chlase activity was found in both leaf tissues, but major activity was found for chl b degradation, except in *A. japonica*, suggesting that an alternate chl degradation pathway may also be involved. Otherwise, evidence indicated that the chloroplast anatomical structure and the chl ratio (a:b) were similar in green and non-green areas of the variegated plant leaves (Fooshee and Henny, 1990). However, the intact chloroplast in the variegated leaves is dramatically reduced (Sangsiri et al., 2007). Our data revealed that the chlase activity was significantly higher in most of the non-green tissues of selected plants (Table 2). It implies that the chlase in green and non-green areas of the variegated leaves may be existed in the differential abundance based on different levels of chloroplasts, or may be processed in different levels. It has been proposed that not only the activity of chlase was latent due to spatial distribution of chlase and chl in the chloroplast (Schoch and Brown, 1987), but also the posttranslational processing of chlase was required to form mature active chlase (Azoulay-Shemer et al., 2008). Our data also reveal that chlase may be processed to mature active form in the envelope membrane of chloroplast (Fig. 1; Table 3). Furthermore, the chlase may be differentially expressed with different isoforms. Emerging evidence indicated that chlase isozymes existed in the same species, and their expression may vary among tissues (Tsuchiya et al., 1997). In Fig. 1, two strong immunoblot signals derived from processing of *Pm*CLH1 in the leaves of *P. macrocarpa* were detected. Some minor addition signals were also detected. This may be caused by nonspecific binding of polyclonal antibody or cross-reactivity of antibody with the other chlase isoforms. Further work needs to be conducted to examine these possibilities.

5. Conclusions

Our study findings are novel in comparing chlase activities between green and non-green tissues of the leaves in variegated plants. Different variegated plants displayed variations in their chlase systems, and the differential expressions of each genotype were associated with the intracellular localization of chlase. These results help explain why green sectors of variegated plants contain high quantities of chl, while non-green sectors have little or no chl. Our findings could lay a foundation for researchers interested in genetics and physiological studies of chlase.

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