Pheophytin Formation with Senescence in Stored Japanese Bunching Onion

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Abstract

The shelf life of Japanese bunching onion (*Allium fistulosum* L.; JBO genome FF) leaves diminished with yellowing as a consequence of chlorophyll (Chl) degradation, which seems to be the main factor in yellowing of stored JBO leaves. The aim of this study was to investigate pheophytin (Phy) formation as a Chl derivative during Chl degradation in stored JBO leaves. A series of alien monosomic addition lines (AMALs, FF+1A to FF+8A) of JBO, with extra chromosomes from shallot (*A. cepa* L., Aggregatum group; genome AA) was used as supportive materials to study the formation of Chl *a* derivatives and the activity of Chl-degrading enzymes during storage. Chl contents in FF+3A and FF+5A decreased greatly during storage at 25°C, whereas in FF+4A the reduction in Chl content was the lowest compared to the control, FF. In JBO leaves, during Chl degradation, the presence of Phy *a* was prominent as Chl derivatives, which was not recorded in other horticultural crops. Moreover, the activities of Chl-degrading enzymes, especially Mg-dechelation, also progressively increased during storage at 25°C. The formation of Phy *a* in stored JBO leaves was further investigated. By incubating the reaction mixture of crude enzyme extract and Chl *a*, Phy *a* was formed by Mg-dechelating action. Especial significant was the greater formation of Phy *a* in FF+3A than FF and FF+4A. Electron microscopic observation elucidated the formation of plastoglobules (Pgs) in chloroplasts and its movement from chloroplast to vacuole in JBO. A large number of Pgs in vacuole of FF+3A might relate to the high Phy *a* formation with Chl degradation. These findings suggest that Chl *a* could be degraded in part, through Phy *a*, and the vacuole along with the chloroplast might be important sites for Chl degradation in JBO during storage.

INTRODUCTION

Yellowing is the main factor in quality deterioration of stored Japanese bunching onion (JBO, *Allium fistulosum* L.; FF). Our previous studies showed that Chl degradation is the reason for yellowing in JBO leaves during storage (Dissanayake et al., 2008a).

Chl degradation is considered to be started by hydrolysis of Chl *a* into chlorophyllide (Chlide) *a* and phytol, catalysed by chlorophyllase (Chlase), (Amir-Shapira et al., 1987) and then, as the second step, elimination of Mg from Chlide *a* to produce pheophorbide (Pheide)

a, in a reaction catalysed by Mg-dechelation (Shioi et al., 1996). Finally, Pheide *a* is decomposed to produce fluorescent Chl catabolites. These reactions, which are thought to be the main pathway for Chl *a* degradation, occur in the chloroplast. Tang et al. (2000) showed the formation of pheophytin (Phy) *a* by Mg-dechelatase in *Ginkgo biloba* leaves. Previous studies on Chl degradation in JBO leaves showed that Phy *a* was present at higher levels than any other Chl derivatives and was considered the main Chl *a* derivative in JBO leaves (Dissanayake et al., 2008a). The degradation process of Chls to form Phy *a* is still not clearly clarified and demands further study to elucidate the pathway for Chl degradation.

In this study, Chl degradation and formation of Phy *a* was studied using 8 alien monosomic addition lines (AMALs) of JBO (Shigyo et al., 1996), as supportive material.

MATERIALS AND METHODS

Plant Material

Healthy mature leaf blade tissue from JBO and eight different AMALs (FF+1A to FF+8A) were harvested and stored in the dark at 25°C for 3 days in perforated polyethylene bags (0.03 mm thick; 38 cm x 26.5 cm) with two 6 mm holes.

Chlorophyll Content

Chl contents of leaves were determined spectrophotometrically (U-2001; Hitachi, Tokyo, Japan) using *N*, *N*-dimethylformamide.

Chlorophyll-Degrading Enzyme Assays

A 300 mg leaf acetone powder from FF (control), FF+3A and FF+4A were stirred separately for 1 h at 0°C in 7.5 ml 10 mM phosphate buffer (pH 7) containing 50 mM KCl and 0.12% Triton X-100 for Chl-degrading peroxidase and Mg-dechelatase assays. For Chlase assay, 10 mM phosphate buffer (pH 7) contained 0.6% CHAPS was used. Then, the extracts were filtered through two layers of Miracloth (Calbiochem) and centrifuged at 16000 × *g* for 15 min at 4°C. The supernatant was used as the crude enzyme extract.

For Chlase activity the reaction mixture contained 0.5 ml of crude enzyme solution, 0.1 ml of 1% CHAPS, 0.2 ml of Chl *a* acetone solution (100 µg Chl *a*) and 0.5 ml of 0.1 M phosphate buffer (pH 8). The mixture was incubated in a water bath at 25°C for 1 h and the reaction was stopped by adding 4 ml of acetone. The remaining Chl *a* was extracted with 4 ml of hexane. The activity was based on the increase in absorbance by Chlide *a* at 667 nm in acetone layer.

To determine Mg-dechelation activity, in one method the reaction mixture contained 0.2 ml of crude enzyme solution, 0.3 ml of 98 nM chlorophyllin (Chlin) *a* and 0.75 ml of 50 mM Tris-HCl buffer (pH 7). Activity was measured at 35°C by following the change in optical density (OD) at 686 nm. In the other method the reaction mixture contained 0.25 ml of 30 µM Chlide *a*, 0.75 ml of 10 mM phosphate buffer (pH 7) and 0.2 ml of crude enzyme solution. Activity was measured at 35°C by following the change in OD at 535 nm.

For Chl-degrading peroxidase activity, the reaction mixture contained 0.2 ml of Chl *a* acetone solution (100 µg Chl *a*), 1% Triton X-100, 0.1 ml of 5 mM *p*-coumaric acid, 1.5 ml of 0.1 M citrate/0.2 M phosphate buffer (pH 4.5), 0.2 ml of enzyme solution and 0.1 ml of

0.3% hydrogen peroxide to determine Chl-degrading peroxidase activity. Activity was determined spectrophotometrically by measuring the decrease in Chl *a* at 668 nm at 25^{o}C.

HPLC Analyses for Chlorophyll Derivatives Formation

The reaction mixture, containing 0.5 ml of leaf crude enzyme solution, 0.2 ml of Chl *a* acetone solution (100 µg Chl *a*) and 0.5 ml of 0.1 M phosphate buffer (pH 8), was incubated in a water bath at 2 ^{o}C for 1 h to analyse Chl *a* derivatives by HPLC at 665 nm.

Preparation for Transmission Electron Microscopy

Leaf tissues of 2 mm x 2 mm in size were fixed with 2.5% glutaraldehyde (EM grade; Taab laboratories, UK) in 0.05 M sodium phosphate buffer (pH 7.0) for 3 h at room temperature on a rotator. After washing with the same buffer for 2, leaf tissues were post-fixed with 1% osmium tetroxide (MERCK, Darmstadt, Germany) at 5^{o}C. Samples were dehydrated in a 50-100% ascending ethanol series for 15-20 min per ethanol concentration. After two 1 h rinses in 100% propylenoxide, specimens were embedded in Spurr's resin. Ultra-thin sections were cut using a Sorvall Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc. Norwalk, Connecticut, USA), placed on copper mesh grids and stained with 2% aqueous uranyl acetate and 0.3% lead citrate. Specimens were observed with a JEOL JEM-1200 EX II transmission electron microscope operating at 80 kV (JEOL Ltd, Tokyo, Japan).

RESULTS AND DISCUSSION

Chlorophyll *a* Changes During Storage

Chl contents in FF+3A and FF+5A decreased greatly during storage at 25°C, whereas in FF+4A the reduction was lowest (Table 1). This identified that the FF+3A and FF+5A as fast Chl-degrading lines, whereas FF+4A as the slow Chl-degrading line.

Chlorophyll-Degrading Enzyme Activities

Chlase activities significantly increased in FF and FF+3A compared to day 0, but not in FF+4A (Fig.1A). Chl-degrading peroxidase activity in FF+3A increased significantly day 1 to day 3, but FF+4A had no significant differences except day 2 (Fig.1B). Mg-dechelation activity detected using Chlin *a* (Fig.2A) and Chlide *a* (Fig.2B) as substrates showed that Mg-dechelation activity in FF+3A significantly increased day 0 to day 3, but not in FF+4A.

Despite the increase in Chlase activity in FF+3A and FF during storage, there were no significant differences in activity between the FF+3A and FF+4A (Fig.1A). Therefore, it was obvious that rapid yellowing in FF and FF+3A was not only by Chlase activity as no yellowing showed in FF+4A during 3 day storage.

These findings suggest that the peroxidase activity also has a significant role in Chl degradation in JBO leaves as most of other horticultural crops (Yamauchi and Watada, 1991).

The significant increase in Mg-dechelation activity with leaf yellowing in FF and FF+3A indicated that Mg-dechelation was involved in yellowing of JBO (Fig.2A and Fig.2B). Two types of Mg-dechelation activities have been distinguished; one associated with a heat-stable low-molecular-weight compound, metal-chelating substance (MCS), and the other catalysed by an enzyme protein (Shioi et al., 1996). The Mg-dechelating protein acts only on

the artificial substrate Chlin *a*, whereas the MCS removes Mg from both substrates **(**Kunieda et al., 2005). These findings suggest that MCS might also be involved in removing Mg from Chlide *a* in JBO. Moreover, in JBO leaves the formation of Phy *a* was observed during storage, but the involvement of Mg-dechelation on Phy *a* formation needs more clarification.

Pheophytin *a* Formation in Stored Japanese Bunching Onion Leaves

In FF, FF+3A and FF+4A, Phy *a* formation from Chl was lower than that of Chlide *a* on day 0 (Fig.3). On day 3, however, the formation of Phy *a* was two times higher than that of Chlide *a* in FF+3A, whereas two times lower in FF+4A. These results suggest that Phy *a* was formed from Chl *a* by Mg-dechelation.

However, with high Chl degradation, formation of Phy *a* was not recorded except in few plants (Tang et al., 2000; Amir-Shapira et al., 1987). In the present study, the formation of Phy *a* was prominent in stored JBO leaves and enhanced especially in FF+3A, compared to the formation of Chlide *a* on day 3 of storage (Fig.3). This indicated that the increase in Mg dechelation activity concomitantly with yellowing of leaves in FF+3A could be the reason for Phy *a* formation in FF+3A as well as in FF and FF+4A. Further, together with an increment in Phy *a* (Fig.3), an increment in Mg dechelation activity during storage, especially in FF+3A (Fig.2A and 2B), indicated that there might be a possible involvement of Mg dechelation in formation of Phy *a* from Chl *a*.

Transmission Electron Microscopic Observation of senescing leaves

Many small Plastoglobules (Pgs), were observed in chloroplasts of FF, FF+3A and FF+4A (Fig.4). Leaf cells on day 0 also showed formation of many Pgs in chloroplasts. Enlarged Pgs were accumulated in vacuoles, especially in FF+3A than FF and FF+4A on day 0. Grouping small droplets of Pgs were clearly observed in chloroplasts and large bodies of Pgs were observed in cytoplasm and especially in vacuoles, after protrusion of enlarged Pgs from chloroplasts through cytoplasm to vacuoles (Fig.4 (A) and (B)).

Barely visible and dissociated thylakoids in chloroplasts of JBO leaves indicated that chloroplasts have already started degradation from day 0. This is further confirmed with the presence of large amounts of Pgs within the chloroplasts because Pgs may serve as storage pools for breakdown thylakoids constituents containing Chl (Biswal and Biswal, 1988; Steinmüller and Tevini, 1985). Chl degradation in JBO leaves started from leaf tips and progressed towards the base as evidenced by the pattern of leaf yellowing. The leaf tissues we used in this study were within 10 cm of leaf tips, where the degradation of chloroplasts has already started. Therefore, Pgs content seems to be high in leaf cells with degradation of thylakoids. However, even though chloroplast structure has already degraded, JBO leaves did not show yellowing on day 0 within 10 cm. This raised speculations about having green colour in leaves within 10 cm. Therefore, it can be assumed that the Chl pigment was likely to be localized in Pgs (Guiamét et al., 1999). Therefore, Chl in Pgs of JBO leaves might be also present. Neveretheless, presence of Chl in isolated Pgs was considered by some scientists as contamination (Steinmüller and Tevini, 1985). Therefore, in our future study, elucidation of the presence of Chl in Pgs of JBO leaves is required.

In Japanese bunching onion, the presence of numerous Pgs within vacuoles explained a movement of Chl pigments from senescing chloroplasts and Chl in Pgs seems to be

degraded in the vacuole. Presence of Mg-dechelating substances could be possible in vacuoles as it has been reported that peroxidase (Takahama, 1992) and possibly Chlase (Tsuchiya et al., 1999) are found in vacuoles. During Chl degradation in stored JBO leaves, the formation of Phy *a* was prominent (Fig.3). This indicates that Mg-dechelating substances in vacuoles are possibly involved in forming Phy *a* from Chl *a*. Low pH conditions in vacuoles (5.0-5.5) (Taiz, 1992) could also formed Phy *a* from Chl *a*. By these reasons, we demonstrate that Chl *a* could be degraded forming Phy *a* within the vacuole in JBO leaves.

Our early studies also showed that the Phy *a* present in stored JBO leaves with Chl degradation and hence Phy *a* was the main Chl derivative in JBO plants during Chl degradation (Dissanayake et al., 2008b). The formation and movement of numerous Pgs from chloroplasts to vacuoles could explain Phy *a* formation in JBO plant.

It can be concluded that Chl degradation in JBO progresses primarily through Chlide *a* and Pheide *a* to colourless Chl catabolites, as in many plant species. In addition to that, Chl *a* is also degraded by Chl-degrading peroxidase to OHChl *a* and then to colourless catabolites. Further, more importantly Chl *a* could also be degraded, forming Phy *a* as the main Chl *a* derivative in JBO leaves partly by Mg-dechelation and partly by the acidic nature of the vacuole, a secondary site of Chl degradation in JBO leaves. Thus, Chl in JBO may be degraded in two organelles both the chloroplast and vacuole.

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Tables

Table 1 Changes in chlorophyll (Chl) a contents during 3 d storage at 25º C in Japanese bunching onion and its eight monosomic addition lines (FF+1 to FF+8).Mean±SD(n=3).

Plants	Chl *a* content (mg $100g^{-1}$FW)			
	Day 0	Day 1	Day 2	Day 3
FF	82.9±10.85	72.7±8.85	51.3±13.16	51.3±9.57
FF+1A	81.7±0.36	75.8±8.43	58.6±4.89	43.3±21.90
FF+2A	47.4±3.18	43.2±6.23	43.3±3.39	33.5±3.46
FF+3A	73.0±2.69	63.4±9.26	49.2±3.73	30.4±0.61
FF+4A	87.6±3.88	89.3±0.66	79.0±9.06	76.5±2.54
FF+5A	66.0±5.14	57.2±4.82	45.6±13.69	34.2±1.21
FF+6A	93.5±5.64	87.3±4.92	73.4±15.45	58.6±5.41
FF+7A	57.0±2.63	56.1±0.62	48.3±2.49	34.3±1.38
FF+8A	67.9±10.17	72.4±3.47	55.3±1.81	53.0±16.03

Figures

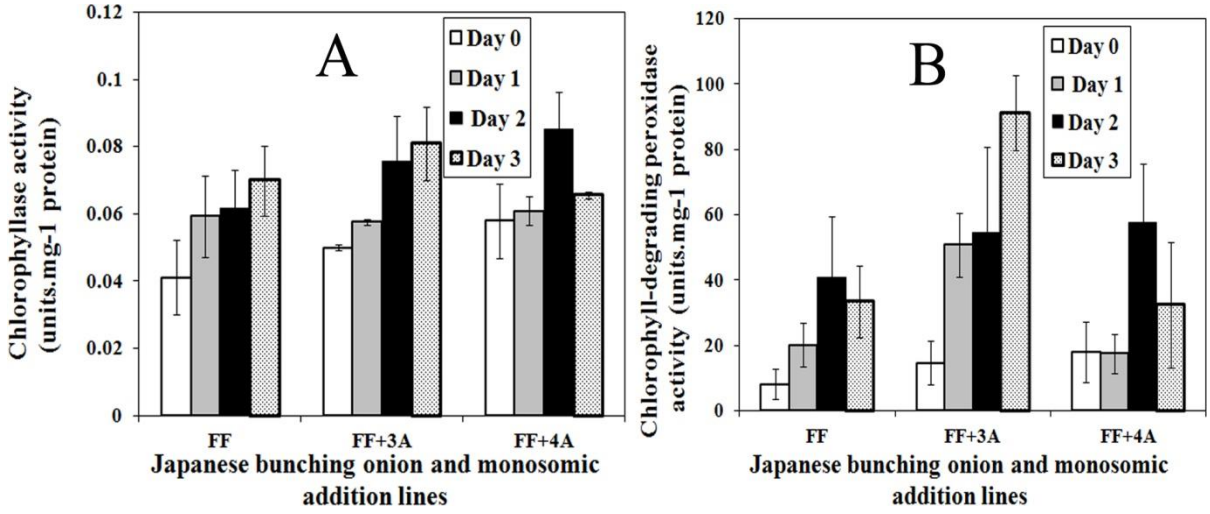

Fig. 1. Chlorophyllase (A) and Chlorophyll-degrading peroxidase (B) activity activity in FF, FF+3A and FF+4A during storage at 25°C. Vertical bars represent average values with ±SD.(n=3).

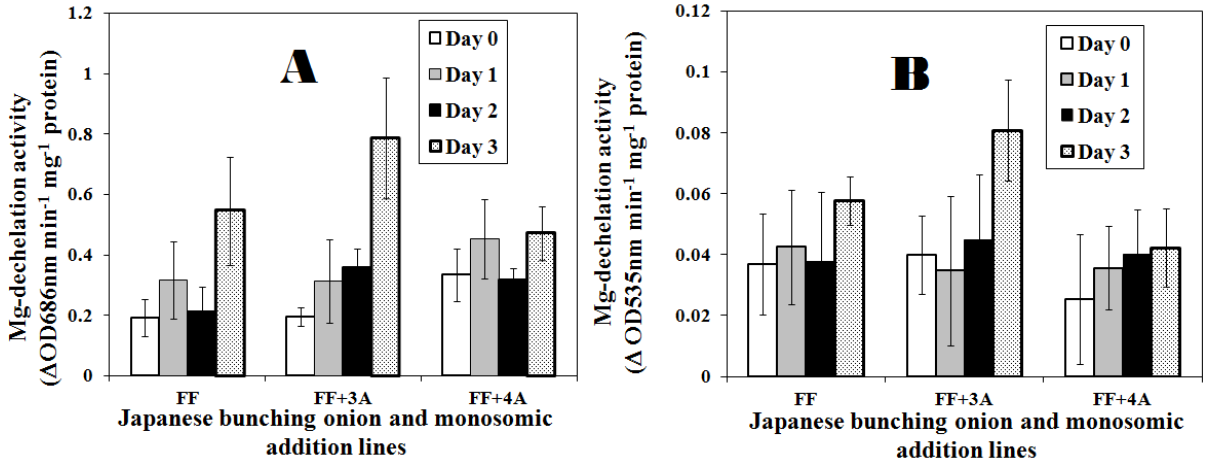

Fig. 2. Mg-dechelation activity in FF, FF+3A and FF+4A during storage at 25°C. Chlorophyllin a used as substrate (A) and Chlorophyllide a used as substrate (B) . Vertical bars represent average values with ±SD.(n=3).

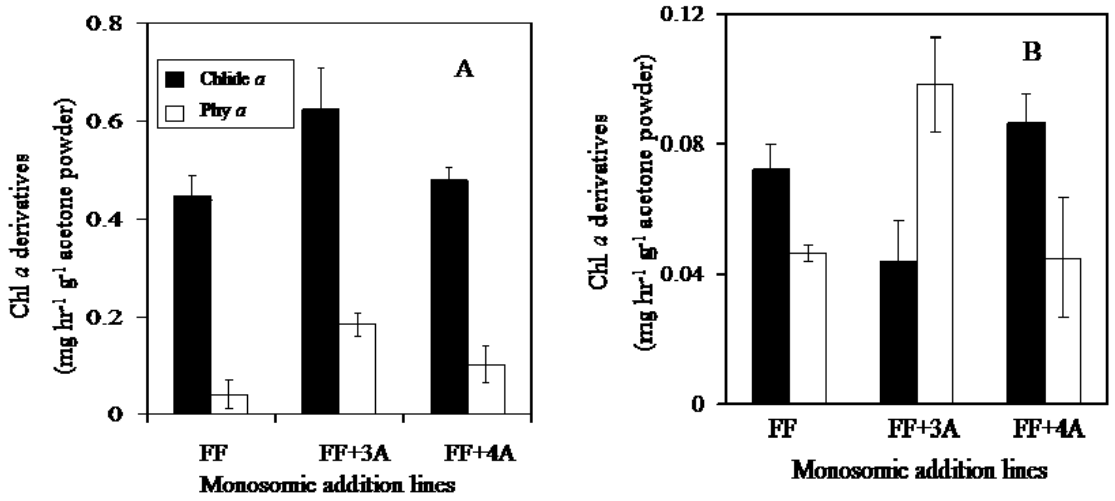

Fig. 3. Chl *a* derivatives formation during incubation of the reaction mixture containing the crude enzyme (from FF, FF+3A and FF+4A) and Chl *a* at 25°C. (A) day 0 storage; (B) day 3 storage; vertical bars represent average values with ±SD.(n=3).

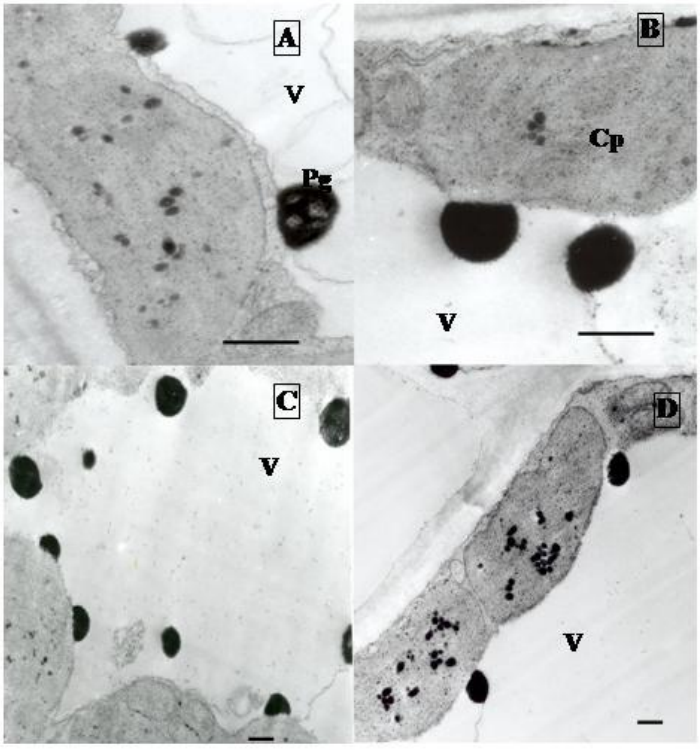

Fig. 4. Electron micrographs of (A) FF day 0; (B) FF+3A day 0; (C) FF+3A day 3; (D) FF+4A day 0; (Pg) plastoglobule; (V) Vacuole; (Cp) Chloroplast. Bars represent 1μM.