

Postharvest Chlorophyll Degradation in Japanese Bunching Onion (*Allium fistulosum* L.)

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ABSTRACT

Japanese bunching onion (*Allium fistulosum* L.; JBO), or welsh onion, is an important green vegetable in East Asia and to a certain extent in the USA. The shelf life of JBO tends to diminish with loss of green leaves mainly due to yellowing as a consequence of chlorophyll (Chl) degradation, which seems to be the main factor in loss of green leaves associated with yellowing in JBO. The mechanism of Chl degradation in stored JBO leaves has been elucidated using eight JBO (genome FF) lines with single alien chromosomes from shallot (genome AA) in each line (FF+1A to FF+8A). JBO leaves stored at 25°C show rapid Chl degradation, while at 4°C retained their greenness throughout the storage period. JBO lines containing 3A and 5A (FF+3A and FF+5A) shallot chromosomes show rapid Chl degradation during storage at 25°C, whereas JBO lines with 4A (FF+4A) shallot chromosome show slow Chl degradation. Pheophytin (Phy) *a*, chlorophyllide (Chlide) *a*, pheophorbide (Pheide) *a* and 13²-hydroxychlorophyll (OHChl) *a* were detected as main derivatives of Chl *a*. Levels of Chl derivatives diminish significantly at 25°C but not at 4°C during storage. However, with Chl degradation, formation of Phy *a* is prominent in stored leaves especially in FF+3A at 25°C. Further, Chl-degrading enzymes, especially Chl-degrading peroxidase and Mg-dechelation, also progressively increase during storage at 25°C. Thus, these findings suggest that Chl *a* could be degraded, in part, through Phy *a*, as well as Chlide *a* and OHChl *a*, in JBO during storage.

Keywords: alien monosomic addition line, chlorophyll degradation, chlorophyll-degrading enzyme, chlorophyll derivative

Abbreviations: AMAL, alien monosomic addition line; Chl, chlorophyll; Chlide, chlorophyllide; Chlin, chlorophyllin; JBO, Japanese bunching onion; OHChl, 13²-hydroxy chlorophyll; Pheide, pheophorbide; Phy, pheophytin

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INTRODUCTION

Japanese bunching onion (JBO; *Allium fistulosum* L.; n=8; genome FF), also known as welsh onion or green onion and in some countries spring onion (Tindall 1983), is a green leafy vegetable used as a condiment for food, fresh vegetables and cooked vegetables in most of East Asia, especially Japan, China and Korea. Elsewhere it is more rarely grown (Hanelt 1990) as green onion or salad onion, especially in western countries. JBO is widely cultivated from Siberia to tropical Asia and shows the largest morphological variability in China, Korea, and Japan (Inden and Asahira 1990; Haishima *et al.* 1993).

The plants are vigorous and grown as an annual herb, never producing enlarged bulbs as onions. Leaves are hollow, circular in cross section, and 15-30 cm in length. Shoots (tillers) increase by tillering. The number of tillers emerging from a seedling in a growing season mainly depends on the type of cultivar or group. The “Ippon-negi”

group and “Kujou-negi” group in Japan produce 1-5 and 20-30 tillers, respectively, in a growing season (Tindall 1983; Yakuwa 2006).

JBO is also subjected to quality losses during storage with leaf yellowing by chlorophyll (Chl) degradation as in other leafy vegetables (Yamauchi and Watada 1991, 1993). Chl degradation or catabolism occurs in green tissues throughout the plant life-cycle, disintegrating the pigment into colourless compounds to store elsewhere as food sources to support new cell development, and is highly sensitive to both biotic and abiotic stress (Guamét *et al.* 1999; Matile 2000; Thomas *et al.* 2001; Krupinska and Humbeck 2008).

Hydrolysing Chl *a* into chlorophyllide (Chlide) *a* and phytol chain by chlorophyllase is considered the first step of Chl degradation (Willstätter and Stoll 1913; Holden 1961; Shimokawa *et al.* 1978; Amir-Shapira *et al.* 1987; Trebitsh *et al.* 1993). Further, dechelation of Mg from Chlide *a* to form pheophorbide (Pheide) *a* by Mg-dechelataase or Mg-dechelating substances is proposed as the second step of

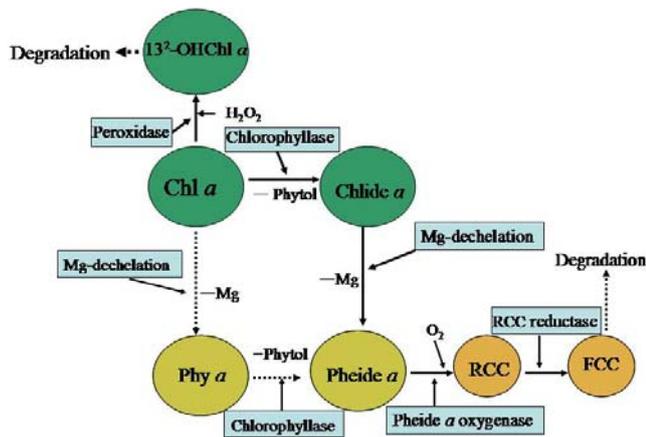


Fig. 1 Putative pathway for chlorophyll degradation in Japanese bunching onion. FCC- fluorescent chlorophyll catabolite; RCC- red chlorophyll catabolite.

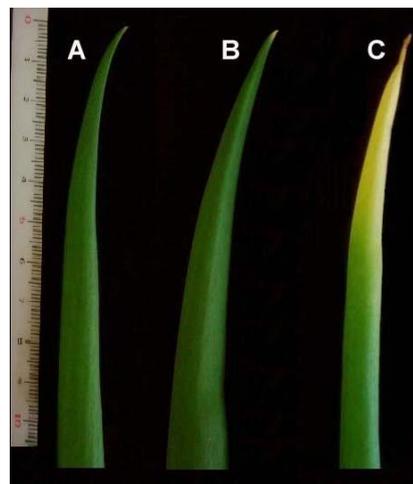


Fig. 2 Japanese bunching onion leaf (A) on day 0; (B) after 3 days' storage at 4°C; (C) after 3 days' storage at 25°C. Scale is in cm.

degradation (Owens and Falkowski 1982; Shimokawa *et al.* 1990; Shioi *et al.* 1991; Langmeier *et al.* 1993; Vicentini *et al.* 1995; Shioi *et al.* 1996). However, instead of removing Mg from Chlide *a*, Tang *et al.* (2000) showed the direct removal of Mg from Chl *a* to form pheophytin (Phy *a*) by Mg-dechelation in *Ginkgo biloba* leaves. Finally, Pheide *a* is decomposed to produce fluorescent colourless Chl catabolites, through red Chl catabolites, involving both Pheide *a* oxygenase and red Chl catabolite reductase (Matile *et al.* 1999). These reactions, which are thought to be the main pathway for Chl *a* degradation, occur in the chloroplast (Matile *et al.* 1999). In addition, Chl *a* is also degraded *in vitro* by peroxidase in the presence of some phenolic compounds and hydrogen peroxide to form 13²-hydroxychlorophyll (OHChl) *a*, an oxidized form of Chl *a* (Huff 1982; Kato and Shimizu 1985; Yamauchi and Minamide 1985; Yamauchi and Watada 1994; Yamauchi *et al.* 2004). Further, during leaf senescence (Kato and Shimizu 1987), seed ripening (Johnson-Flanagan and Spencer 1996), olive fruit ripening (Gandul-Rojas 2004) and storage of vegetables (Yamauchi *et al.* 1999; Funamoto *et al.* 2003), peroxidase activity has been correlated with Chl degradation. However, Chl degradation is a less prominently addressed issue in the plant physiology field compared to the biosynthetic pathway of Chls. Quantitative changes in Chl and degradation products of Chl and/or activity changes of Chl-degrading enzymes have been monitored in vegetables, but the mechanisms of degradation are not clear (Yamauchi and Watada 1991) (Fig. 1).

In this review, we discuss mechanisms of Chl degradation in stored JBO leaves using alien monosomic addition lines of JBO, which have been produced by including single extra chromosomes from shallot (*A. cepa* L. Aggregatum group; n=8; genome AA).

CHLOROPHYLL DEGRADATION IN STORED JAPANESE BUNCHING ONION LEAVES

Chlorophyll and resulting derivative changes

Chl degradation is considered to be the major causal factor in quality deterioration or discolouration of stored JBO during storage (Dissanayake *et al.* 2008a), similar to that of other green vegetables such as broccoli, spinach, parsley and green beans (Yamauchi and Watada 1991; Yamauchi and Watada 1993; Monreal *et al.* 1999; Costa *et al.* 2005). Postharvest yellowing of JBO is initiated at leaf tips and progressively extends towards the base during storage (Dissanayake *et al.* 2008a). The storage life of JBO at 5°C is about 1 week, whereas storage at 25°C causes rapid yellowing and decay of the leaves (Fig. 2). Low temperature storage (at 0°C) and controlled atmospheric conditions

(10% O₂ + 10-40% CO₂) can extend shelf-life, by maintaining the green colour of JBO as well as in spinach, and parsley-like leafy vegetables (Hardenburg *et al.* 1986). In leafy vegetables such as parsley and spinach, Chl content decreases greatly when leaves are stored at 20°C (Yamauchi and Watada 1991, 1993). In broccoli, floret yellowing occurs immediately during storage at 15°C after harvest, and controlled atmospheres (10% CO₂ + 1%O₂) are needed to maintain quality during storage (Yamauchi and Watada 1998).

Chlide *a*, Pheide *a*, Phy *a* and OHChl *a* are present as the main derivatives of Chl *a* in JBO leaves (Dissanayake *et al.* 2008a). However, levels of individual catabolites in leaves are very different from each other (Fig. 3). The high content of Phy *a* indicates that it is the main Chl derivative in JBO leaves. The level of Phy *a* in JBO leaves was 4 times higher than that of Chlide *a* (Dissanayake *et al.* 2008a). However, there has been no previous record of the formation of Phy *a* as a main derivative in leafy vegetables. Although Amir-Shapira *et al.* (1987) mentioned the formation of Phy *a* in stored parsley leaves, the observations of Yamauchi and Watada (1993) indicated absence of Phy *a* in stored parsley leaves. Tang *et al.* (2000), however, showed that Phy *a* was the only derivative in yellowing leaves of *G. biloba*. Furthermore, Phy *a* accumulation in Langra mango fruit has also been reported (Janave and Sharma 2006). JBO leaves stored at 25°C showed a trend of reduction in Phy *a* content on day 3 of storage concomitant with leaf yellowing,

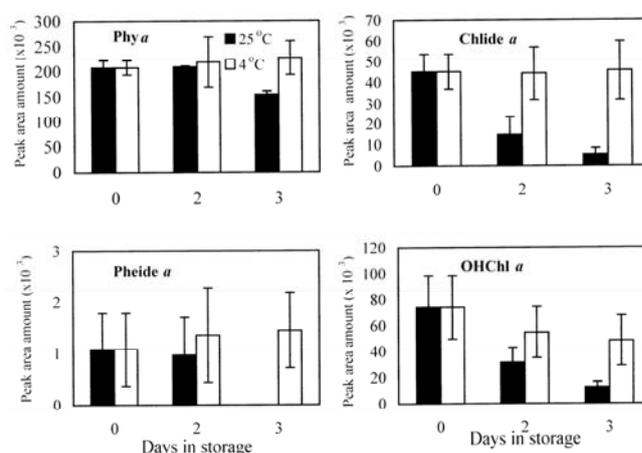


Fig. 3 Chlorophyll *a* derivatives in Japanese bunching onion leaves during storage at 4 and 25°C. Vertical bars represent average values with standard deviation (n=3).

whereas no reduction was evident in leaves stored at 4°C. The formation of Phy *a* in stored leaves is still controversial, as it is not described in the main pathway of Chl degradation (Vicentini *et al.* 1995; Hörtensteiner 2006). Therefore further investigation of the formation of Phy *a* in stored JBO leaves is necessary.

In almost all leafy vegetables, especially in spinach (Yamauchi and Watada 1991), parsley (Yamauchi and Watada 1993), broccoli (Yamauchi and Watada 1998) and radish cotyledons (Akiyama *et al.* 2000), levels of OHChl *a* are reduced during storage similar to that in JBO stored at 25°C, whereas in JBO leaves stored at 4°C, no significant reduction was found. This means that degradation of OHChl is high in leaves with rapid Chl degradation at 25°C. Levels of Chlide *a* in parsley, spinach and radish cotyledons, first increased slightly and then reduced during storage. In JBO, Chlide *a* level is continuously reduced during storage at 25°C but does not change in leaves stored at 4°C. In general, all Chl *a* derivatives in JBO diminish concomitantly with leaf yellowing during 3 days of storage at 25°C, whereas no significant reductions were recorded at 4°C.

Chlorophyll-degrading enzyme activity changes

In JBO, the activity of chlorophyllase increases slightly during storage at 25° and 4°C, but there are no significant differences between the two storage conditions (Dissanayake *et al.* 2008a). In different plant species, the activity of chlorophyllase differs during senescence. In spinach (Yamauchi and Watada 1991) and barley (Sabater and Rodríguez 1978), the activity of chlorophyllase is enhanced during senescence, while a decrease in chlorophyllase activity has been found in green beans (*Phaseolus vulgaris*) (Fang *et al.* 1998) and broccoli florets (*Brassica oleracea*) (Funamoto *et al.* 2002). In JBO, this means that the increase in chlorophyllase activity could not be directly involved in Chl degradation during senescence.

The activity of Chl-degrading peroxidase at 25°C increases significantly on day 2 of storage and remains at the same level on day 3 (Dissanayake *et al.* 2008a). This is concomitant with the yellowing of leaf blades during storage. At 4°C, there is neither a significant decrease in Chl *a* nor an increase in Chl-degrading peroxidase activity during storage. In most crops, including horticultural crops, Chl declines during senescence concomitantly with an elevation of peroxidase activity (Yamauchi and Watada 1991; Ketsa *et al.* 1999; Gong and Mattheis 2003). These findings (Dissanayake *et al.* 2008a) suggested that the activity of peroxidase could play a significant role in Chl degradation in JBO leaves during storage.

Mg-dechelation activity in stored JBO leaves was determined by two different methods, one using Chlorophyllin (Chlin) *a*, an artificial substrate, and the other using Chlide *a*, a native substrate (Dissanayake *et al.* 2008a). The activity of Mg-dechelation, using Chlin *a* as substrate, in leaves stored at 25°C increased greatly from day 1 to day 3, while there were no differences in Mg-dechelation activity in leaves stored at 4°C. Using the method with Chlide *a* as substrate, the activity also showed the same trends for 25 and 4°C storage, and the activity at 25°C was higher than at 4°C on day 3. The increase in Mg-dechelation activity with leaf yellowing at 25°C indicates that Mg-dechelation was involved in yellowing of JBO leaves by catalyzing the degradation of Chlide *a* to Pheide *a* during storage. Furthermore, an increase in Mg-dechelation activity was also reported in Chl degradation during ripening of strawberry fruit (Costa *et al.* 2002). In contrast, a decrease in Mg-dechelation activity was found in oilseed rape cotyledons (Vicentini *et al.* 1995), indicating that there are differences in the behavior of Mg-dechelation activity among plants during senescence. Two types of Mg-dechelation activities have been distinguished, one associated with a heat-stable low molecular weight compound known as a metal-chelating substance (MCS), and the other catalysed by an enzyme protein (Shioi *et al.* 1996; Hörtensteiner 2006). The Mg-

dechelating protein acts only on the artificial substrate Chlin *a* but not on the native substrate Chlide *a*, whereas the MCS removes Mg from both substrates (Suzuki and Shioi 2002; Kunieda *et al.* 2005). Therefore these findings suggest that a MCS might be also involved in removing Mg from Chlide *a* in JBO. Moreover, Tang *et al.* (2000) showed the direct involvement of Mg-dechelation in converting Chl *a* to Phy *a* in yellowing *G. biloba* leaves, whereas Shioi *et al.* (1996) reported that the release of Mg occurred only from the de-phytylated compound, Chlide *a*. However, in our study (Dissanayake *et al.* 2008a) the formation of Phy *a* was observed during storage, indicating that Mg-dechelation might act directly on the removal of Mg from Chl *a* to form Phy *a* in JBO leaves. Further study is necessary to characterize the MCS in JBO leaves.

ATTEMPT AT CLARIFICATION OF CHLOROPHYLL DEGRADATION OF JBO USING ALIEN MONOSOMIC ADDITION LINES

Chlorophyll content throughout the year

More interestingly, an experiment using 8 alien monosomic addition lines (AMALs) of JBO showed different patterns of Chl content throughout the year (Dissanayake *et al.* 2008b). AMALs of JBO, developed by Shigyo *et al.* (1996), each has a single extra chromosome from shallot (*A. cepa* L. *Aggregatum* group). There are 8 lines, designated FF+1A to FF+8A. Chl content in the leaves of JBO and all AMALs differs significantly from month-to-month throughout the year. On average, most plants exhibit a relatively high total Chl concentration in winter, especially in January, and a relatively low Chl concentration in March and April. Moreover, the Chl content of JBO is influenced by the alien chromosomes as well as by seasonal changes.

Higher Chl content was found in FF+4A and FF+5A in winter than in FF, which suggests that chromosomes 4A and 5A from shallot had a greater influence on Chl formation than the other chromosomes. However, chromosome 2A from shallot had no considerable effect on Chl formation in JBO. FF+2A was identified morphologically as the plant with the lightest green leaf blades among other AMALs and JBO (Shigyo *et al.* 1997), which could be due to low Chl content of its leaves. Thus, the Chl contents of the AMALs are affected by different alien chromosomes from shallot, suggesting that gene expression in JBO may be modified by the alien genes (Shigyo *et al.* 1997). However, further investigation is needed to elucidate the principle behind the effects of shallot chromosomes on Chl formation or suppression in JBO leaves.

Chlorophyll and resulting derivative changes

There is large variation in the rate of Chl degradation among AMALs during storage (Dissanayake *et al.* 2008b). In FF+3A and FF+5A, Chl content declines sharply during storage, while it was lowest in FF+4A compared to the JBO FF. FF+3A and FF+5A can be considered rapid Chl-degrading lines, and FF+4A is a slow Chl-degrading line (Fig. 4). This indicates that shallot chromosomes, especially 4A, could be used to improve the postharvest life of JBO. However, shallot chromosomes 3A and 5A did not extend the postharvest life of JBO. Thus, the addition of each shallot chromosome to JBO might affect the expression of enzymes involved in the biosynthesis and/or degradation of Chl *a*, providing an apparently useful background for studying mechanisms of Chl degradation in JBO leaves.

Using FF, FF+3A, and FF+4A, the formation of Chl *a* derivatives was determined during storage (Dissanayake *et al.* 2008b). Chlide *a*, Pheide *a*, OHChl *a*, and Phy *a* are all derivatives of Chl *a* in JBO and its AMALs. In addition to the formation of Chlide *a* and OHChl *a*, formation of Phy *a* appears to be involved in Chl degradation in JBO during storage. The Phy *a* level in FF+3A (a fast Chl-degrading line) increases during storage, while in FF+4A (a slow Chl-

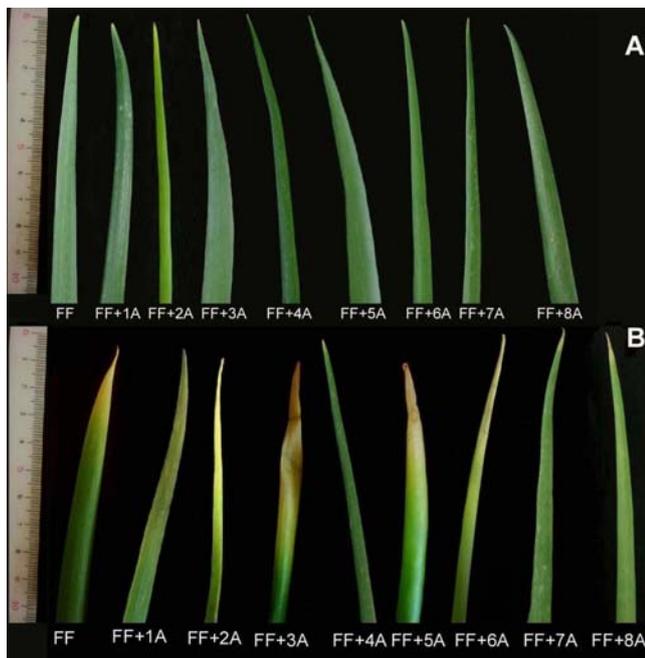


Fig. 4 (A) Leaves of Japanese bunching onion (FF) and AMALs (FF+1A ~ FF+8A) on day 0; (B) Leaves of Japanese bunching onion (FF) and AMALs (FF+1A ~ FF+8A) after 3 days of storage at 25°C. The scale is in cm.

degrading line), this derivative changes slightly (**Fig. 5**).

Meanwhile, OHChl *a* is also found in high quantities, but OHChl *a* levels decline during storage in FF+3A (a fast Chl-degrading line) and in FF (control) as in other horticultural crops such as spinach (Yamauchi and Watada 1991), parsley (Yamauchi and Watada 1993), broccoli (Yamauchi and Watada 1998), and radish cotyledons (Akiyama *et al.* 2000). In contrast, an increase of OHChl *a* was found in excised barley and bean leaves during storage (Maunder *et al.* 1983). In the slow Chl-degrading FF+4A line, OHChl *a* levels show a slight change as compared to a rapid reduction observed in FF+3A. There are clear differences in changes in derivatives of Chl *a*, especially Phy *a* and OHChl *a*, between fast and slow Chl-degrading lines during storage at 25°C. Thus, these findings indicate that fast Chl-degradation in FF+3A could be involved in the accumulation of Phy *a* and further degradation of OHChl *a* to colourless, low molecular weight compounds with high Chl-degrading enzyme activities, whereas slow Chl-degradation in FF+4A might result in constant levels of both derivatives during storage.

Chlide *a* decreases gradually in all plants during storage, but Pheide *a* shows almost no change and is found at very low levels. In stored parsley leaves, Yamauchi and Watada (1993) noted that Chlide *a* levels decrease with yellowing after a temporary increase, similar to those noted in spinach leaves (Yamauchi and Watada 1991) and radish cotyledons (Akiyama *et al.* 2000). On the other hand, in broccoli florets, Chlide *a* level decreases considerably without showing any increment during storage (Yamauchi and Watada 1998). Chlide *a* levels are much lower compared to Phy *a* and OHChl *a* in JBO leaves during storage and changes in Chlide *a* levels follow almost the same pattern in FF, FF+3A, and FF+4A, irrespective of high or low Chl-degradation. Pheide *a* levels in FF, FF+3A, and FF+4A leaves also remain unchanged during storage. These findings indicate that Chlide *a* might be degraded through Pheide *a* to colourless low molecular weight compounds without the accumulation of intermediates. Further work is needed to clarify any changes in Chl-degrading enzymes such as chlorophyllase and Mg-dechelataase.

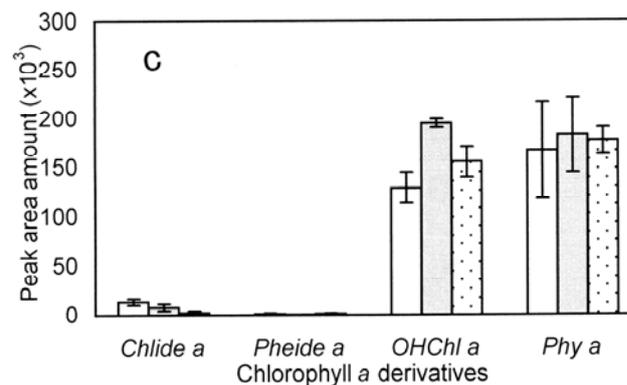
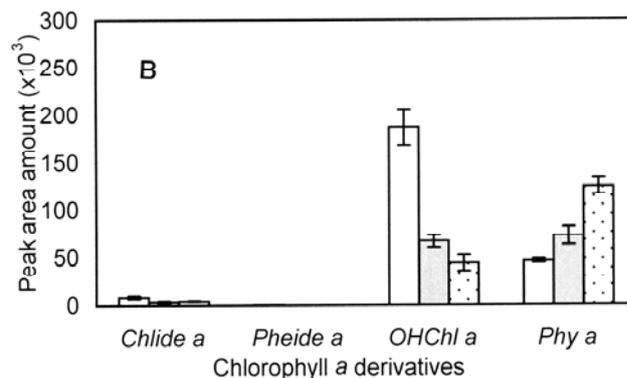
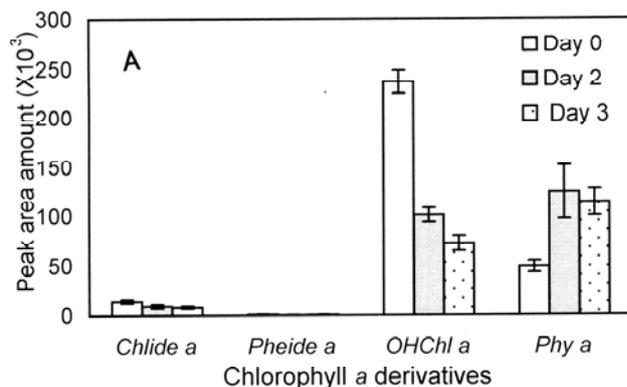


Fig. 5 Chlorophyll *a* derivatives formation in (A) Japanese bunching onion (FF), (B) FF+3A and (C) FF+4A during 3 days storage at 25°C.

CONCLUSION

The Chl content of JBO leaves is influenced differentially by extra chromosomes from shallot, particularly with regard to Chl degradation. Chl degradation is accelerated in JBO leaves stored at 25°C. Mainly all derivatives of Chl *a* decrease during 3 days of storage at 25°C, suggesting that the catabolites might be rapidly converted into their following forms. Activities of Mg-dechelation and Chl-degrading peroxidase are significantly enhanced during storage at 25°C, indicating that these two enzymes have major roles in Chl degradation in stored JBO leaves. The presence of Chlide *a*, OHChl *a*, Pheide *a* and Phy *a* as derivatives of Chl *a* in stored JBO and its AMALs leaves, suggest that Chl *a* could be degraded, in part, through Phy *a*, as well as *via* Chlide *a* and OHChl *a* in JBO and its AMALs during storage (**Fig. 1**). Further study is necessary to elucidate the formation of Phy *a* in senescing JBO leaves as formation of Phy *a* during Chl degradation is still controversial.

ACKNOWLEDGEMENTS

We thank Dr. Cindy B.S. Tong, University of Minnesota, for her gracious and critical reading of the manuscript. This work was

supported in part by 'Knowledge Cluster Initiative', MEXT and a Grant-in-Aid for Scientific Research (No. 13760024) from the Japanese Society for the Promotion of Science.

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