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Influence of Cold Hardening on Chlorophyll and Carotenoid in *Chlorella vulgaris*

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Chlorella vulgaris C–27 has developed freezing tolerance by hardening at 3 ºC for 24 hour. It was reported that many genes which encode proteins related stress response, storage, protein synthesis and metabolism including ζ–carotene desaturase increase at transcriptional levels. We focus on the change of photosynthesis pigments, and assessed the effects of the cold acclimation on chlorophyll and carotenoid in the hardened and unhardened cells of *C. vulgaris* C–27 and *C. vulgaris* C–102 (a chilling–sensitive strain). Cold hardening at 3 ºC for 24 hour caused a slight reduce of the chlorophyll content in *C. vulgaris* C–27, but did a significant reduction of that in *C. vulgaris* C–102. In chlorella cells, six major carotenoids, *i.e.*, β–carotene, α–carotene, lutein, zeaxanthin, violaxanthin and neoxanthin, were detected by an original method for carotenoid determination of *C. vulgaris* C–27 and *C. vulgaris* C–102. It was possible to separate lutein and zeaxanthin by HPLC using cholester column. The carotenoid composition was highly influenced by the cold hardening. It was elucidated that total carotenoid was little affected by hardening at 3 ºC, but zeaxanthin content increased with decrease of violaxanthin content. On the other hand, incubation at 25 ºC after freeze–thaw restored the altered levels of both pigments to pre–hardening levels. This result suggests that freezing tolerance of *C. vulgaris* C–27 induced during cold acclimation have a close involvement of change in xanthophyll cycle which plays a significant role to relieve oxidative stress at freezing and thawing.

INTRODUCTION

Cold is a major environmental limitation to crop productivity. Some plants acquire freezing tolerance when they are exposed to non–freezing low temperature. It was known that many specific morphological, physiological and molecular changes occur with cold acclimation including changes in composition of lipid membranes, increases in total soluble protein content and increases in levels of sugars and proline. It was reported that cold acclimation is associated with changes in gene expression (Thomashow *et al*., 1999). This observation led to the hypothesis that changes in gene expression were probably responsible for some of the biochemical and physiological changes that occurred in response to low temperature and were likely to contribute to an increase in freezing tolerance (Heather *et a*l., 2006). When plants are exposed to a temperature below the normal physiological range, they exhibit various responses and their photosynthetic performance has declined (Close *et al*., 2001). This cold–induced photoinhibition is the continuous decrease in the photochemical efficiency of photosystem Ⅱ (PSII) located in thylakoid membranes with accompanying a regulation of light energy absorption and dissipation (Hola *et al*., 2007). However, long–term cold acclimation decreases susceptibility to photoinhibition (Bravo1, 2007) by causing several metabolic alterations, such as a release of light–harvesting antennae from the PSII reaction center, an activation of the xanthophyll cycle (Watoson *et al*., 2004), accumulation of xanthophylls, and in some cases an additional increase in the biosynthesis of carotenoids (Montane *et al*., 1999).

C. vulgaris has been used as a convenient eukaryotic model to study the development of freezing tolerance in plants during cold acclimation (Joh *et al*., 1995). Hardened cells of C. *vulgaris* C–27 are able to survive slow freezing down to –20 ºC. Several factors related to the development of freezing tolerance, such as accumulation of some late embryogenesis abundant (HIC6 and HIC12) proteins (Honjoh *et al*., 2000), increases in the expression levels of two fatty acid desaturase genes involved in unsaturated fatty acid contents (Suga *et al*., 2002), and an increase in the activity of glucose 6–phosphate dehydrogenase (Honjoh *et al*., 2007), were demonstrated. Alternatively, Machida *et al*. attempted to identify novel hardening–induced genes from *C. vulgaris* C–27, and revealed that ζ–carotene desaturase (ZDS), which catalyzed the reaction from ζ–carotene to lycopene in carotenoid biosynthesis, exhibited remarkable increase in transcripts over 100 times greater than those of unhardened cells (Machida *et al*., 2008). However, information about photosynthetic pigment concerned with a photoprotection in *C. vulgaris* C–27

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during cold acclimation is still lacking. In this study, we examined relative changes in levels of total chlorophyll and total carotenoid, and in composition of carotenoid, especially, xanthophyll cycle pigments, of *C. vulgaris* C–27 cells hardened at 3 °C and grown after freeze–thaw.

MATERIAL AND METHOD

Plant material

Chlorella vulgaris Beijerick IAM C–27, hardy strain, was obtained from the Algal Culture Collection of the institute of Applied Microbiology, The University of Tokyo. *C. vulgaris* C–27 cells were grown in MC medium at 25 °C with bubbling of air containing 1.3% CO_2 in a 16–h–light / 8–h–dark photoperiod (photosynthetic photon flux density of about 250 mol/m^{2}s), about 1.0×10^{10} cells per liter, to synchronize the same stage cells. Therefore, the survival rate of hardened cells was maximum at the L_2 stage (ripening phase) in the life cycle (Hatano *et al.*, 1976). The L_2 stage cells were obtained from the culture on nine hours later after a second or third cycle of 16 hour–light, 8 hour–dark regime, and were used in frost hardening test.

Hardening and freezing tolerance test

A culture of L_2 stage cells (1.0×10^9 cells per liter) was directly hardened by incubation at 3 °C for 24 hours. During hardening, it was aerated with air containing 1.3% CO_2 and kept in the light (photosynthetic photon flux density of about 250 mol/m^{2}s).

Hardened and unhardened cell were collected by centrifugation at 2,500×*g* for 5 min at 3 °C and 25 °C, respectively. After removing supernatant, the collected cells resuspended with MC medium at a concentration of 5×10^{10} cells per liter. Two milliliters of the cell suspension were frozen at –20 °C for 20 hours. The frozen cell suspension was thawed at room temperature with shaking, and subsequently diluted to a concentration of 2×10^9 cells per liter by MC medium. The cells were incubated at 25 °C on continuous light, and cell number was counted with a Thoma hemacytometer. Freezing tolerance was evaluated by the growth rate of cells after thaw.

Extract of carotenoids from *Chlorella vulgaris* C–27

Extraction of the chlorophyll and carotenoids from chlorella cells was carried out by a modification of the method described previously (Yamada *et al.*, 2002). Chlorella samples were obtained by centrifuging of the cultured cells at 2,500×*g* for 5 min and were frozen by liquid nitrogen and freeze–dried, followed by stored at −80 °C. The freeze–dried cells were vortexed in 1 ml of methanol with zirconia ball (3.0 mm in diameter) for 2 min. After centrifugation of the mixture at 1,200×*g* for 3 min at 4 °C, the supernatant was collected. The centrifuged pellets were repetitively washed with another 1 ml of methanol until the cells became colorless. After filtration of the combined methanol solution, its absorbance at 665 and 650 nm was measured, and total chlorophyll content in this solution was calculated according to the method described by MacKinney (MacKinney, 1940). The extract of the colored carotenoids was carried out as follows, to the remaining methanol solution (8 ml), 1 ml of a 60% (v/v) KOH in water solution was added (the final KOH concentration was 6%), and the mixture was incubated at 60 °C for 20 min. The colored carotenoids were partitioned into 10% diethyl ether (v/v) in petroleum ether (boiling point 60 to 70 °C), and the ether solution was successively washed with water and brine. After concentrating the ether solution, the residue was kept at −80 °C under nitrogen gas until the HPLC analysis.

Chromatographic analysis of carotenoids

The extract sample was resuspended in 200 μl of cold acetone. Total carotenoid amount was determined from this extract solution by absorbance at 445 nm and calculated with average extinction coefficient (E: 1%, 1 cm) of 2500.

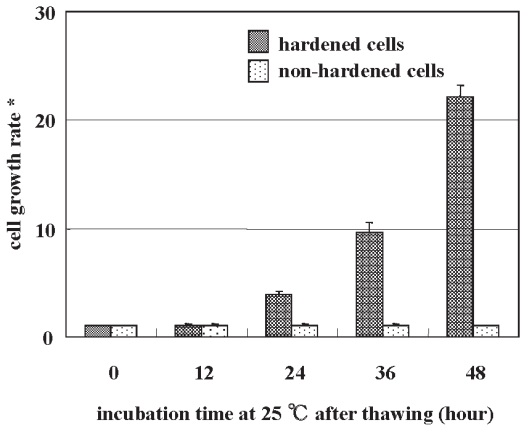
Chromatographic analyses were performed using Simadzu co. (Kyoto, Japan) reversed–phase HPLC system (model: Prominence) with photodiode array detector (PDA). Chromatography was carried out by original method using Cosmosil Cholester column (4.6 mm× 250 mm i.d.; 5 μm particle size, nacalai tesque co. Japan). The mobile phase consisted of two components: solvent A, acetonitrile/methanol/2–propanol/H_2O/dichloromethane (81.6 : 9.6 : 4.8 : 3.0 : 1.0; v/v %), and solvent B, acetonitrile/methanol/2–propanol/ethyl acetate (59.5 : 7.0 : 3.5 : 30.0; v/v %), The carotenoids were eluted at flow rate of 1.0 ml/min, using a linear gradient. The gradient program was as follows: % A/min, 0/0, 10/7, 20/12, 100/17, 100/ 40. The extracts was scanned by PDA in rang 250–550 nm. Peaks were detected and integrated at a 450 nm for every caroetnoid. The carotenoids were identified by comparing their retention time and visible spectrum with standard.

RESULT AND DISCUSSION

It was reported that the hardened cells of *C. vulgaris* C–27 exhibited greatly enhanced antifreeze capability after cold hardening at 3 °C for 24 hours. Therefore, a growth of hardened cells after freeze–thaw was determined by counting cells (Fig. 1). The hardened cells rapidly proliferated within 24 hours after freeze–thaw, but unhardened cells completely failed to proliferate. The effect of hardening at 3 °C on the growth of *C. vulgaris* C–27 cells was investigated (Fig. 2). The cell number was increased up to 7.0–fold by the incubation at 25 °C for 24 hours, whereas it remained unchanged during cold hardening at 3 °C. In general, plants respond and adapt to cold stresses through various biochemical and physiological processes, thereby acquiring freezing tolerance. These results indicate that cell divisions were inhibited at 3 °C, but biochemical change with some stress related gene expression occurred in the *C. vulgaris* cells during this cold hardening.

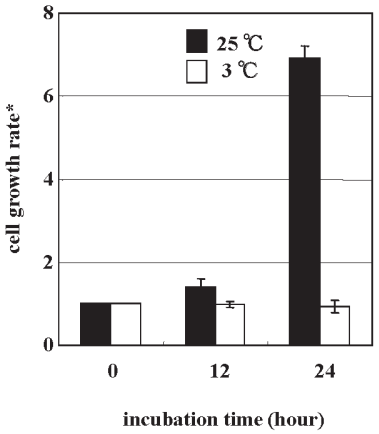
When the cells of *C. vulgaris* C–27 were exposed at a low temperature at 3 °C, slight color change to yellow-

ish green was observed. Total chlorophyll and total carotenoid amounts in the cold hardened cells and in the incubated cells at 25 °C after freeze–thaw were analyzed (Fig. 3). Under hardening condition, total chlorophyll amount was slightly decreased. After freeze–thaw, it was promptly recovered at same level, and kept in similar extent even with cell growth. Total carotenoid decreased as well as total chlorophyll during cold acclimation, but it recovered slowly compared to total chlorophyll amount after freeze–thaw. In contrast, total chlorophyll and carotenoids in unhardened cells of *C. vulgaris* C–27 were dramatically decreased in the first 12 hours after freeze–thaw (Fig. 4). It is known that carotenoids directly quench both triplet chlorophyll and singlet oxygen generated by photoinhibition under stress conditions in plant, and the freezing condition inevitably leads to the generation of active oxygen molecules. These data implies that cold acclimated cells of *C. vulgaris* C–27 have some functions in quenching of active oxygen molecules and can reduce a damage of cell membranes and photosynthetic pigments after freeze–thaw. The remained pigments in *C. vulgaris* C–27 after cold hard-



* cell growth rate =(number of cell at each time / number of cell at starting)

Fig. 1. Survival of hardened or non–hardened cell of *C. vulgaris* C–27 after freeze– thaw. Data are the means of four replicates. Vertical lines are S.D.



* cell growth rate =(number of cell at each time / number of cell at starting)

Fig. 2. Cell growth of *C. vulgaris* C–27 L_2 cells at 3 °C or 25 °C. Data are the means of four replicates. Vertical lines are S.D.

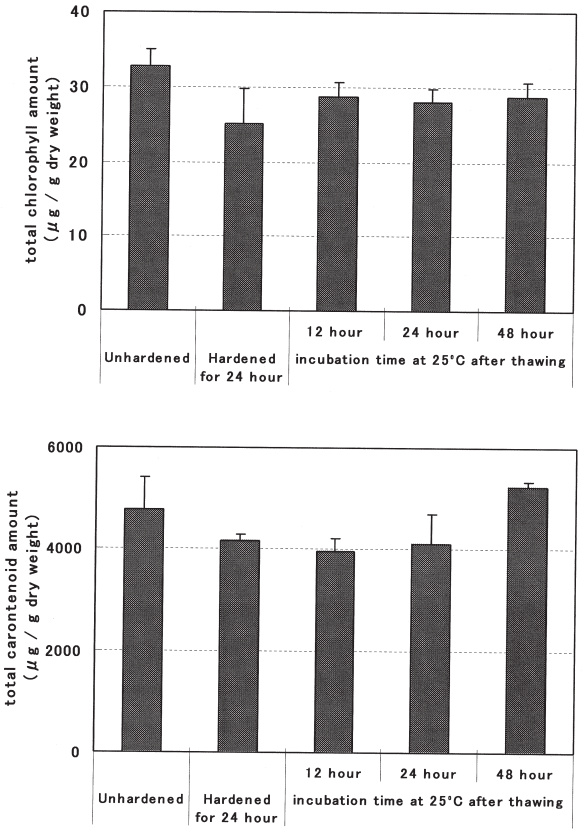


Fig. 3. Changes in total chlorophyll and total carotenoid amounts of cells hardened and incubated at 25 °C after freeze–thaw in *C. vulgaris* C–27. Data are the means of four replicates. Vertical lines are S.D.

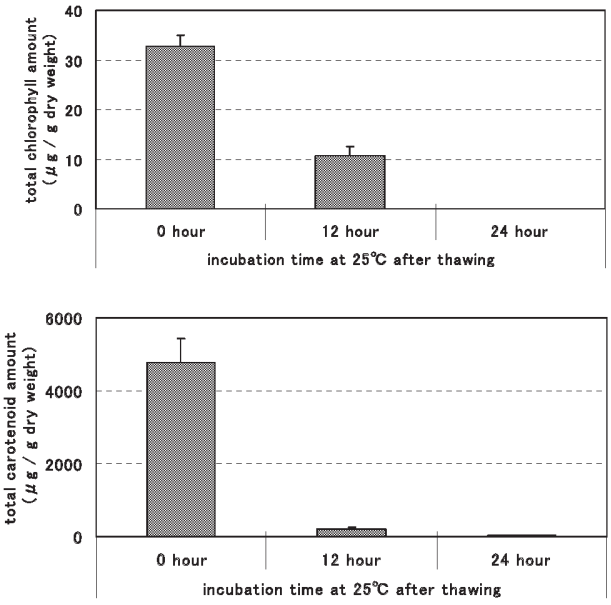


Fig. 4. Changes in total chlorophyll and total carotenoid amounts of non–hardened cells during incubation at 25 °C after freeze–thaw in *C. vulgaris* C–27. Data are the means of four replicates. Vertical lines are S.D.

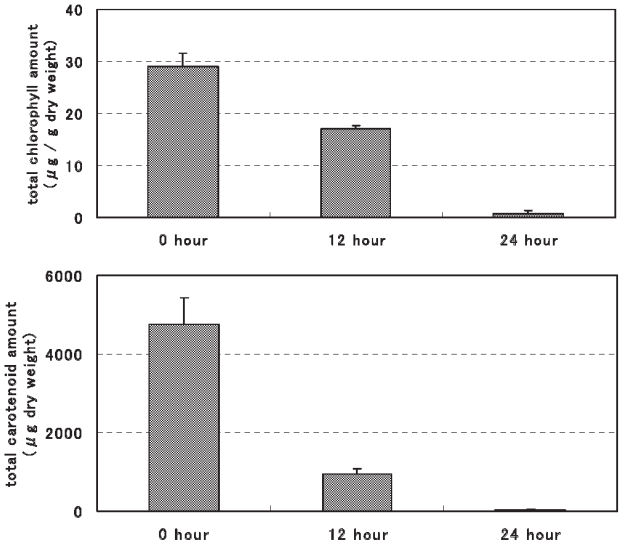


Fig. 5. Changes in total chlorophyll and total carotenoid amounts during incubation at 3 ℃ in *C. vulgaris* C–102. Data are the means of four replicates. Vertical lines are S.D.

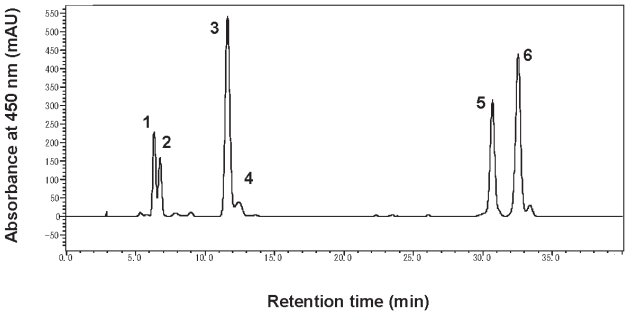


Fig. 6. HPLC chromatogram detected at 450 nm of carotenoids extracted from *C. vulgaris* C–27. Peaks : 1, neoxanthin; 2, violaxanthin; 3, lutein; 4, zeaxanthin; 5, α–carotene; 6, β–carotene.

ening may play an important role in this freezing tolerance.

In addition, we examined the changes during incubation at 3 ℃ in total chlorophyll and total carotenoids of *C. vulgaris* C–102, which is chilling–sensitive strain and is not able to survive after freeze–thaw (Fig. 5). Unlike the case of *C. vulgaris* C–27 cells, the cold stress at 3 ℃ caused intense decreases of both pigments in *C. vulgaris* C–102. The estimation of freezing tolerance of plants is not constitutively expressed (Zhou *et al*., 2004), but it is induced in response to low temperature exposure (cold acclimation). Therefore, It is reasonable to suppose that knowledge of the change in photosynthetic pigments occurs during cold acclimation would allow a better understanding of freezing tolerance.

In rye leaves, total chlorophyll and carotenoid contents increased during cold acclimation at 4 ℃ (Streb *et al*., 1999), but a slight reduction of total carotenoid in *C. vulgaris* C–27 occurred during the hardening. It was known that a composition of carotenoids, especially xanthophylls, is usually influenced by cold hardening (Bravo *et al*., 2007), so we examined the variation in quantity of each carotenoid by HPLC analysis. Six major carotenoids (neoxanthin, violaxanthon, lutein, zeaxanthin, β–carotene, α–carotene) in *C. vulgaris* C–27 and C–102 were detected (Fig. 6), and the total content of six carotenoid was more than 95% of detected carotenoids in sum of peak area equivalent in absobance at 450 nm. The dramatic increase in zeaxanthin content with decreasing of violaxanthin content was caused, but four other carotenoid did not change so much under cold hardening (Table 1). The observed fluctuation during cold hardening from violaxanthin to zeaxanthin has been explained by xanthophyll cycle (Fig. 7). The xanthophyll cycle takes an important role of non–photochemical quenching of excess chlorophyll fluorescence generated by high light irradiation (Havaux *et al*., 2001). It is noted that in high light, violaxanthin is converted into zeaxanthin, via the intermediated antheraxanthin, by the enzyme violaxanthin de–epoxydase. When the light intensity decreases, the interconversion from violaxanthin to zeaxanthin is reversed. The low temperature caused to the photoinhibition linked to photodamege, but xanthophyll cycle was activated by cold hardening and reduce the photoinhibition to relieve intense oxidative stress as well as high light condition.

The each carotenoid content per cell fresh weight and carotenoid composition of *C. vulgaris* C–27 cells

Table 1. Change of each carotenoid content of hardened cells during incubation after freeze–thaw in *C. vulgaris* C–27

	carotenoid content (μg/g dry weight)					
	α–carotene	lutein	β–carotene	zeaxanthin	violaxanthin	neoxanthin
Unhardened	586.4±172.4 (1.00)	1872.7±432.9 (1.00)	984.0±141.9 (1.00)	94.1±23.2 (1.00)	596.4±132.1 (1.00)	633.3±94.5 (1.00)
Hardened for 24 hour	358.7±25.2 (0.61)	1880.0±80.3 (1.00)	913.4±36.5 (0.93)	245.2±10.8 (2.60)	95.5±7.7 (0.16)	561.3±56.7 (0.89)
Incubation at 25 ℃ after thawing for 12 hour	660.4±24.8 (1.13)	1573.1±133.5 (0.84)	741.3±27.4 (0.75)	72.9±1.4 (0.77)	469.4±37.1 (0.79)	507.0±47.1 (0.80)
Incubation at 25 ℃ after thawing for 24 hour	528.5±69.5 (0.90)	1726.8±271.3 (0.92)	778.8±101.4 (0.79)	79.6±9.5 (0.85)	370.1±58.0 (0.62)	659.0±83.5 (1.04)
Incubation at 25 ℃ after thawing for 48 hour	439.3±17.5 (0.75)	2285.6±46.9 (1.22)	970.3±17.7 (0.99)	68.8±1.9 (0.73)	568.9±13.5 (0.95)	846.0±46.6 (1.34)

* Each value is mean of 4 replicates ± S.D.
** Figures in parentheses represent the ratio of each carotenoid to that of unhardened cells.

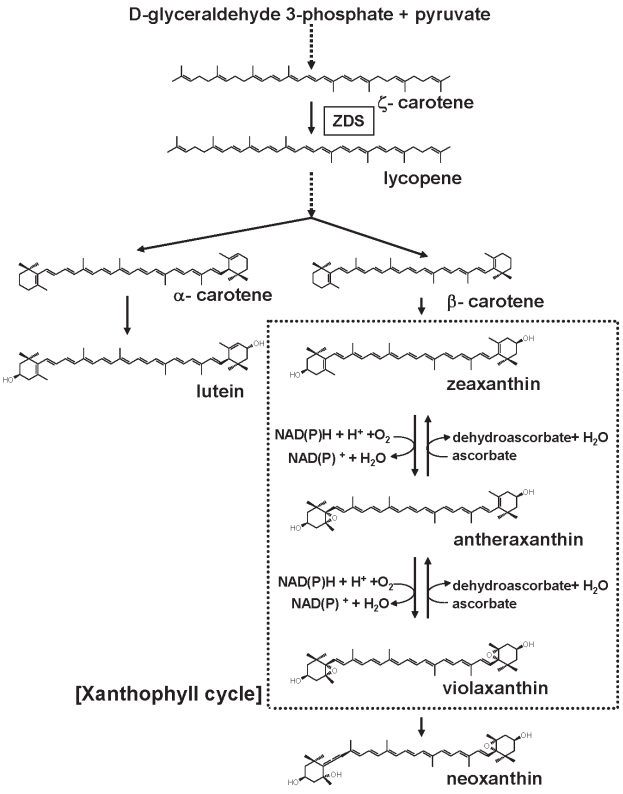


Fig. 7. Carotenoid biosynthesis pathway and xanthophylls cycle in plants.
ZDS; ζ –carotene desaturase.

remained unaltered by the incubation at 25 °C in light, non–hardening condition (data not shown). In contract, the significant increse in zeaxanthin of *C. vulgaris* C–102 cells was not observed during incubation at 3 °C (Table 2). All carotenoid contents of *C. vulgaris* C–102 cells showed the same pattern in notable decreasing. This result shows that incubation at 3 °C is the critical chilling condition caused a marked photoinhibition, further cell injury for *C. vulgaris* C–102.

In spite of a few difference in the amount of total carotenoid between hardened and unhardened cells, both cells differed on the carotenod compositions. (Table 1, Table 3). By the incubation at 25 °C for 12 hours after freeze–thaw, zeaxanthin and violaxanthin contents (per dry weight cells) in hardened cell recovered to pre–hardening levels. However, all carotenoids of unhardened cells were dramatically decreased in the first 12 hours after freeze–thaw. There is a possibility that the xanthophylls cycle in the protection against photo–damage was a critical factor of acquiring freezing tolerance in this case of *C. vulgaris* C–27, because the increasing of zeaxanthin was produced under cold acclimation, but it was not observed in cells of *C. vulgaris* C–102. It was suggested that the regulation of xanthophyll cycle during cold acclimation is essential for freezing tolerance, the cells without cold acclimation become sensitive to severe oxidative stress after thawing.

Our study demonstrated that the essential cold hardening at 3 °C for an acquisition of freezing tolerance in *C. vulgaris* C–27 is accompanied by an increase of zeaxanthin, a slight decrease of total carotenoid and xantophyll cycle pigments. We concluded that freezing tolerance of

Table 2. Change of each carotenoid content during incubation at 3 °C in *C. vulgaris* C–102

	carotenoid content (μg/g dry weight)					
	α–carotene	lutein	β–carotene	zeaxanthin	violaxanthin	neoxanthin
Incubation for 0 hour	334.1±37.6 (1.00)	1768.8±234.0 (1.00)	963.1±83.2 (1.00)	131.3±28.5 (1.00)	433.8±66.1 (1.00)	385.0±25.2 (1.00)
Incubation at 3 °C for 12 hour	41.0±5.6 (0.12)	517.8±66.1 (0.29)	96.1±13.9 (0.10)	27.7±3.8 (0.21)	69.8±9.3 (0.16)	53.3±9.2 (0.14)
Incubation at 3 °C for 24 hour	<3.0 (<0.01)	32.0±4.1 (0.02)	<3.0 (<0.01)	<1.0 (<0.01)	<1.0 (<0.01)	<1.0 (<0.01)

* Each value is mean of 4 replicates ± S.D.
** Figures in parentheses represent the ratio of each carotenoid to that of unhardened cells.

Table 3. Change of each carotenoid content of unhardened cells during incubation after freeze–thaw in *C. vulgaris* C–27

	carotenoid content (μg/g dry weight)					
	α–carotene	lutein	β–carotene	zeaxanthin	violaxanthin	neoxanthin
Incubation for 0 hour	586.4±172.4 (1.00)	1872.7±432.9 (1.00)	984.0±141.9 (1.00)	94.1±23.2 (1.00)	596.4±132.1 (1.00)	633.3±94.5 (1.00)
Incubation at 25°C after thawing for 12 hour	<5.0 (<0.01)	182.6±24.2 (0.10)	<6.0 (<0.01)	6.1±1.1 (0.06)	<5.0 (<0.01)	6.7±1.5 (0.01)
Incubation at 25°C after thawing for 24 hour	<5.0 (<0.01)	32.8±3.3 (0.02)	<6.0 (<0.01)	<1.0 (<0.01)	<5.0 (<0.01)	<1.0 (<0.01)

* Each value is mean of 4 replicates ± S.D.
** Figures in parentheses represent the ratio of each carotenoid to that of unhardened cells.

C. vulgaris C–27 was concerned with not only photochemical quenching effect of carotenoids in hardened cells on some antioxidants generated after freeze–thaw, but also nonphotochemical quenching effect of xanthophyll cycle, which restore the energy balance in photosystem, on photo–oxidative damage after freeze–thaw.

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