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EFFECT OF STORAGE CONDITIONS ON TOTAL CAROTENOID CONTENT IN GOLDEN RICE GRAINS

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ABSTRACT

The amount of total carotenoid in golden rice grains obtained from ten different transgenic golden rice lines (from Taipen 309) were investigated for evaluating stability of total carotenoid under different storage conditions. The reading at OD=475 was used to calculate total carotenoid contents in transgenic golden rice grains. Amounts of total carotenoid in golden rice grains were ranging from 0.81 - 1.37 μg/g of milled grains. Effect of two different storage temperatures on stability of total carotenoid in rice grains was not obviously clear-cut. However, it was observed that the amount of carotenoid content in grains seemed to be more stable when stored at 4°C than being stored at room temperature condition. The experiment clearly demonstrated that, level of total carotenoid content in golden rice grains is statistically reduced after 5 months of storage at the rate of 5 to 10% reduction. There is no or less reduction of total carotenoid content in grains in the first two months of storage.

Key words: Beta-carotene, carotenoid analysis, golden rice, transgenic rice.

INTRODUCTION

The transfer of golden rice project from its inventor to few renowned rice research institutions including Cuu long Delta Rice Research Institute has opened a new wave of breeding efforts to adopt golden-coloured grain trait into their local rice varieties by both conventional breeding and molecular-based methods. Golden rice was developed as a fortified food to be used in areas where there is a shortage of dietary vitamin A. The prototype lines of golden rice were genetically engineered with three beta-carotene biosynthesis genes; *psy* (phytoene synthase), *lyc* (lycopene cyclase) and *crt1*(a bacterial phytoene desaturase) (Ye et. al. 2000) This transgenic rice which was called "golden rice one" or SGR1, under green house conditions, produced up to 1.6 μg of carotenoids per gram of milled grains. Hoa and Bong (2003) had successfully transferred the golden-colored grain trait into several indica varieties and replaced an antibiotic selection system with the use of phosphomannose isomerase as selectable marker. This has helped to resolve several regulatory issues that have slowed the widespread implementation of golden rice technology. Foreseeing the successful implication of golden rice in coming years, we are interested in evaluating the stability of total carotenoid which should reflect the status of beta-carotene in golden rice grains under different storage conditions. This paper described our preliminary results on the effect of two storage temperatures (at 4°C and at room temperatures) on the total carotenoid content in rice grains harvested from greenhouse- grown golden rice progenies for ten transformation events over five months of storage.

MATERIAL AND METHOD:

Seven T4 and three T6 transgenic lines (from Taipen 309) that had visible golden-coloured grains were selected and grown in greenhouse in Dong Xuan season, 2005. The name list of these transgenic events is given in appendix 1. Harvested rice grains were dried under sunshine for few days and then divided into two equal amounts to be kept either at 4°C or at room temperature (around 28°C) for up to five months storage. Every a month, five grams of grains were taken out and dehusked, polished with sand papers to totally remove bran and then crushed in mortar. The very fine ground powder was used for measuring beta-carotene content using a photometry method

as described by Schaub and Beyer (2005). Three replications of 0.3g for each treatment were weighed into a 15ml falcon tube, added with 3ml ethanol: BHT (2,6- Di-tert-butyl-4-methylphenol, 1mg/ml) (one gram of BHT for 1ml of ethanol, prepare fresh daily) and mixed the sample. Samples were incubated at 86^{o}C in water bath for 5 minutes, vortexed for 10 seconds and repeated twice with incubation and mixing. Samples were cooled down on ice, added 3ml petroleum benzene: diethyl ether (2: 1 v/v) and filled up to 14 ml with 1% NaCl solution. Samples were then vigorously shaken and centrifuged for 10 minutes at 1400 x g. The upper phase was transferred to a 2ml-eppendorf and dried the transferred epiphase using a vacuum concentrator rotated at 30^{o}C. Repeated adding 2 ml of petroleum benzene: diethyl ether to 15ml Falcon tube, mixed, centrifuged and transferred to 2ml-eppendorf as described before. The dried 2ml-eppendorf was dissolved in 2ml petroleum benzene and taken for photometry immediately to minimise solvent evaporation. To measure ß-carotene spectrum, the ODs at 465, 470, 475, 480 and 485nm wavelengths were taken to find the peak of carotenoid absorbance. We selected the reading by which almost all non-transformed rice samples from wild type give the reading of zero for calculating the amount of total carotenoid in rice grains. All work was carried out in dim daylight. Lambert-Beer equation was used to calculate the amount of carotenoid in rice samples. Graphs were analysed and drawn using the Statview software (version 5.0; SAS Inc., Carey, NC, USA).

RESULTS:

A range of wavelengths at 465, 470, 475, 480 and 485nm was tested to find the maximum reading at which it could be the nearest point to the peak of carotenoid absorbance. Using grains of non transformed Taipen 309 and golden rice plants for the test, we found out that the reading taken at wavelength of 475 nm gave higher value than that measured at neighboring tested wavelengths for golden rice seeds. The reading at OD=475 for untransformed Taipen 309 was almost zero, whereas other wavelengths gave the reading far from zero (data not shown). Hence we decided to take the reading at OD=475 for estimating the amount of total carotenoid in golden rice grains because this wavelength could allow the calculation of total carotenoid content in rice grains with less contamination by other components.

Table 1 gave levels of total carotenoid contents measured at OD=475 for ten transgenic rice lines numbered from 1 to 10. They were ranging from 0.81 to 1.37 µg carotenoid per one gram of milled grains. It was clearly shown no difference in size and shape of grains between golden rice transgenic and untransformed Taipen 309 plants except for a yellow-coloured grain trait as demonstrated in figure 1 and figure 2 for transgenic events numbered 1 and 3, respectively. Figure 3 presented the graph of total carotenoid contents measured from grains of ten transgenic lines that were stored over five months. General speaking, there was a reductive trend of total carotenoid content after three months of storage at both 4^{o}C and room storage temperatures. Two transgenic events, lines 8 and 10, showed inconsistent variation in total carotenoid content in grains. Five transgenic events numbered 1, 2, 3, 4 and 6 gave significant reduction in total carotenoid contents after four or five months of storage as compared to the total carotenoid measured at the first or second months of storage (ANOVA; $p < 0.05$).

Table 1: Total carotenoid contents in transgenic golden rice grains calculated at OD= 475. At this OD, the readings of non transformed taipen 309 grain samples are almost zero

N0.	Treatment	Carotenoid (μg/g)
1	10F5	1.03
2	10R5	0.88
3	10F4	1.08
4	10R4	0.88
5	10F3	1.01
6	10R3	0.81
7	10F2	1.02
8	10R2	0.96
9	10F1	1.00
10	10R1	0.87
11	9F5	0.93
12	9R5	0.95
13	9F4	1.07
14	9R4	0.88
15	9F3	0.96
16	9R3	0.89
17	9F2	1.06
18	9R2	0.86
19	9F1	1.02
20	9R1	1.02
21	8F5	1.03
22	8R5	0.99
23	8F4	1.18
24	8R4	1.34
25	8F3	1.51

N0.	Treatment	Carotenoid (μg/g)
26	8R3	1.15
27	8F2	1.03
28	8R2	1.37
29	8F1	1.21
30	8R1	0.82
31	7F5	1.04
32	7R5	0.96
33	7F4	1.04
34	7R4	1.06
35	7F3	1.08
36	7R3	1.02
37	7F2	1.03
38	7R2	1.02
39	7F1	1.06
40	7R1	1.06
41	6F5	1.13
42	6R5	1.07
43	6F4	1.02
44	6R4	1.02
45	6F3	1.13
46	6R3	1.00
47	6F2	1.22
48	6R2	1.11
49	6F1	1.18
50	6R1	1.03

N0.	Treatment	Carotenoid (μg/g)
51	5F5	1.15
52	5R5	0.96
53	5F4	1.04
54	5R4	1.09
55	5F3	1.03
56	5R3	1.01
57	5F2	1.05
58	5R2	1.05
59	5F1	1.07
60	5R1	1.06
61	4F5	1.11
62	4R5	1.15
63	4F4	1.15
64	4R4	1.13
65	4F3	1.25
66	4R3	1.02
67	4F2	1.17
68	4R2	1.23
69	4F1	1.19
70	4R1	1.22
71	3F5	1.20
72	3R5	1.10
73	3F4	1.17
74	3R4	1.21
75	3F3	1.17

N0.	Treatment	Carotenoid (μg/g)
76	3R3	1.33
77	3F2	1.26
78	3R2	1.26
79	3F1	1.25
80	3R1	1.23
81	2F5	1.04
82	2R5	1.00
83	2F4	1.15
84	2R4	1.12
85	2F3	1.12
86	2R3	1.05
87	2F2	1.07
88	2R2	1.01
89	2F1	1.11
90	2R1	1.14
91	1F5	1.00
92	1R5	1.04
93	1F4	0.95
94	1R4	1.02
95	1F3	1.02
96	1R3	1.04
97	1F2	1.05
98	1R2	1.04
99	1F1	1.11
100	1R1	1.12

Note for abbreviation of treatment: eg. for 1R3, the first digit denotes for variety numbered from 1 to 10; a letter is for temperature conditions: Room (R) or Fridge (F) temperature of storage; the second digit for months of storage after harvesting, from 1 to 5 months.

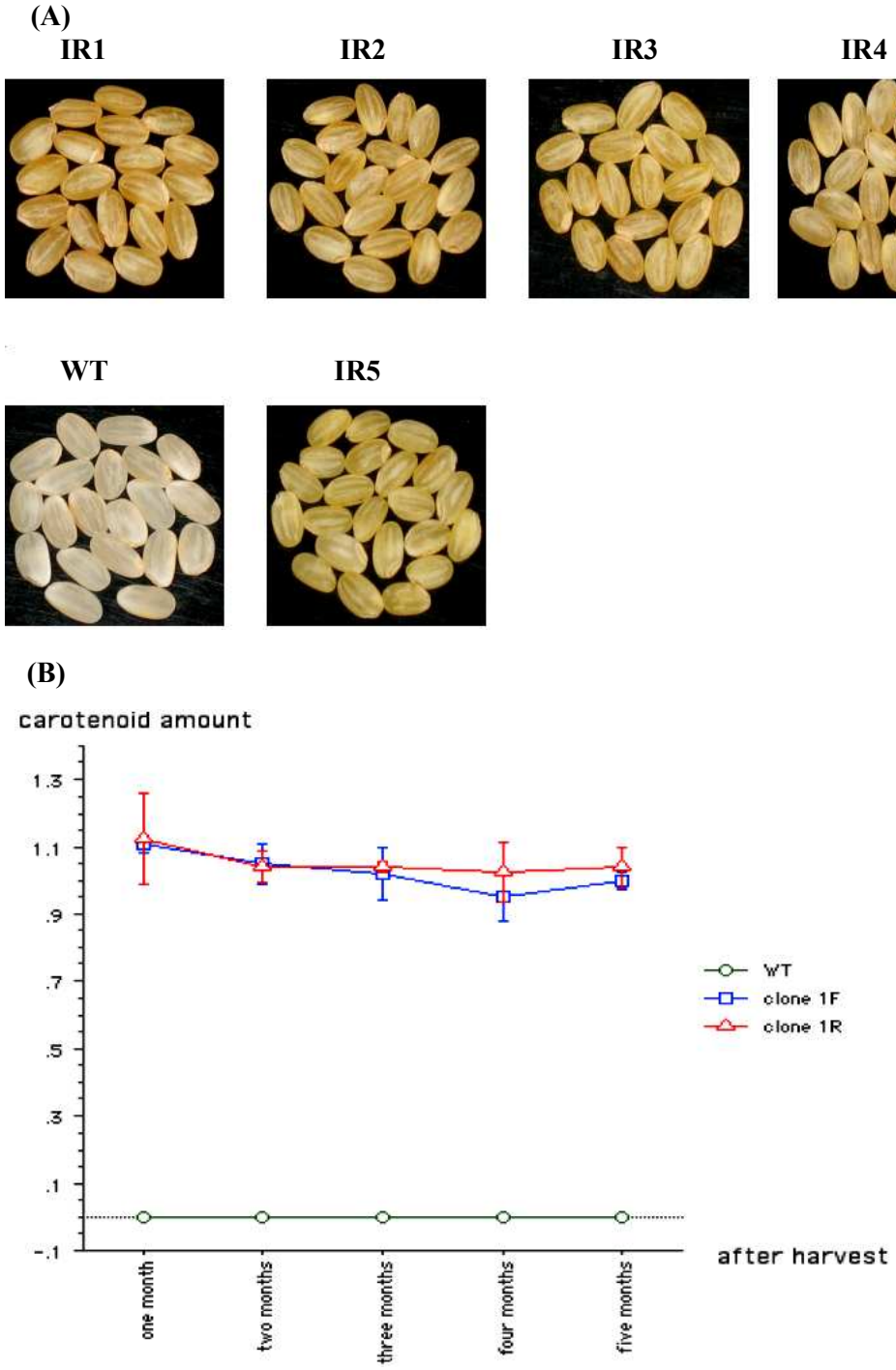

Figure 1: (A) Photos of grain samples of golden rice numbered 1, stored at room temperature over 5 months of storage; (B) Chart presenting total carotenoid contents in grains of golden rice numbered 1 (ANOVA; $p < 0.05$).

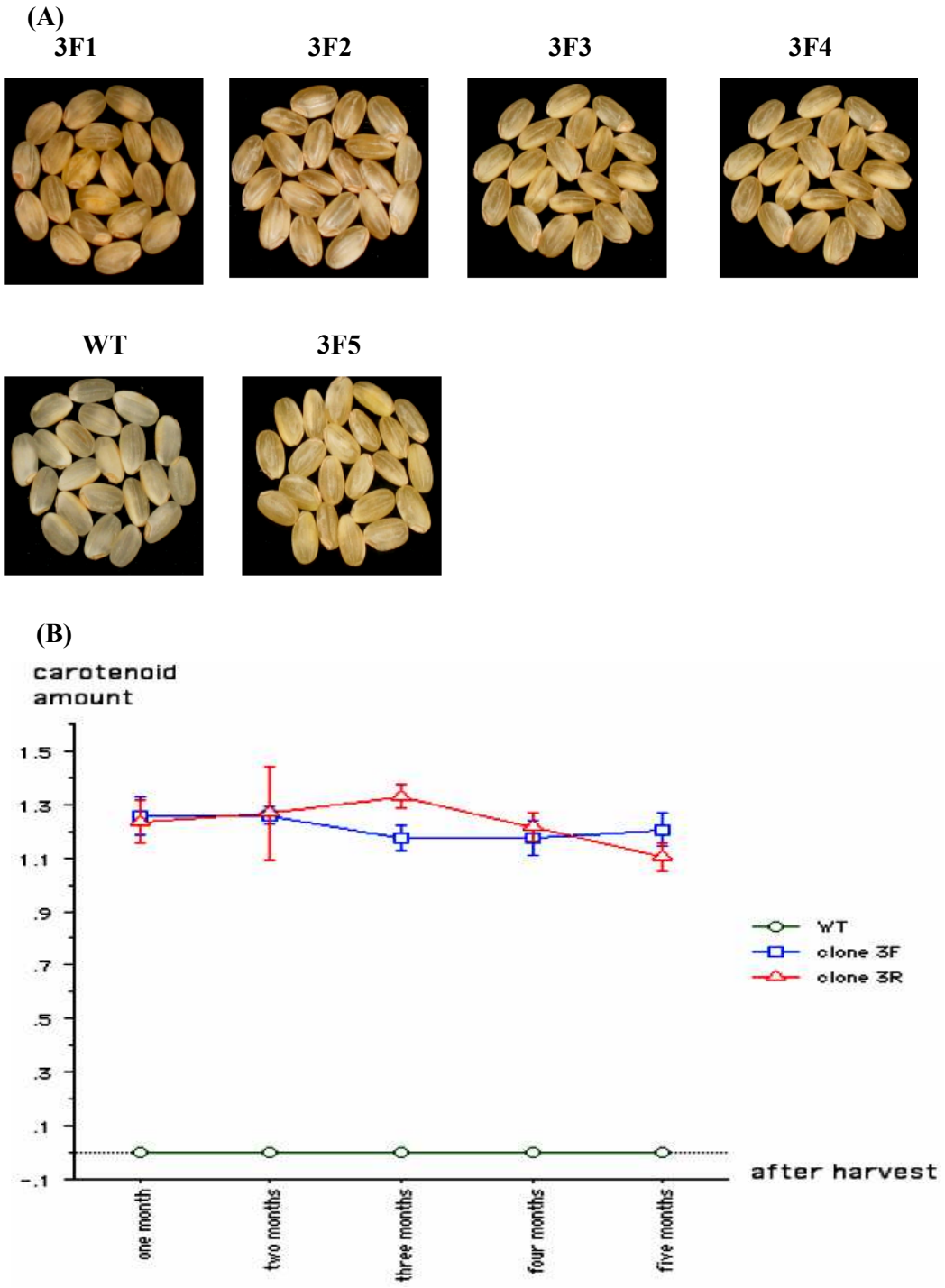

Figure 2: (A) Photos of grain samples of golden rice numbered 3, stored at fridge temperature over 5 months of storage; (B) Chart presenting Carotenoid contents in grains of golden rice numbered 3 (ANOVA; $p < 0.05$).

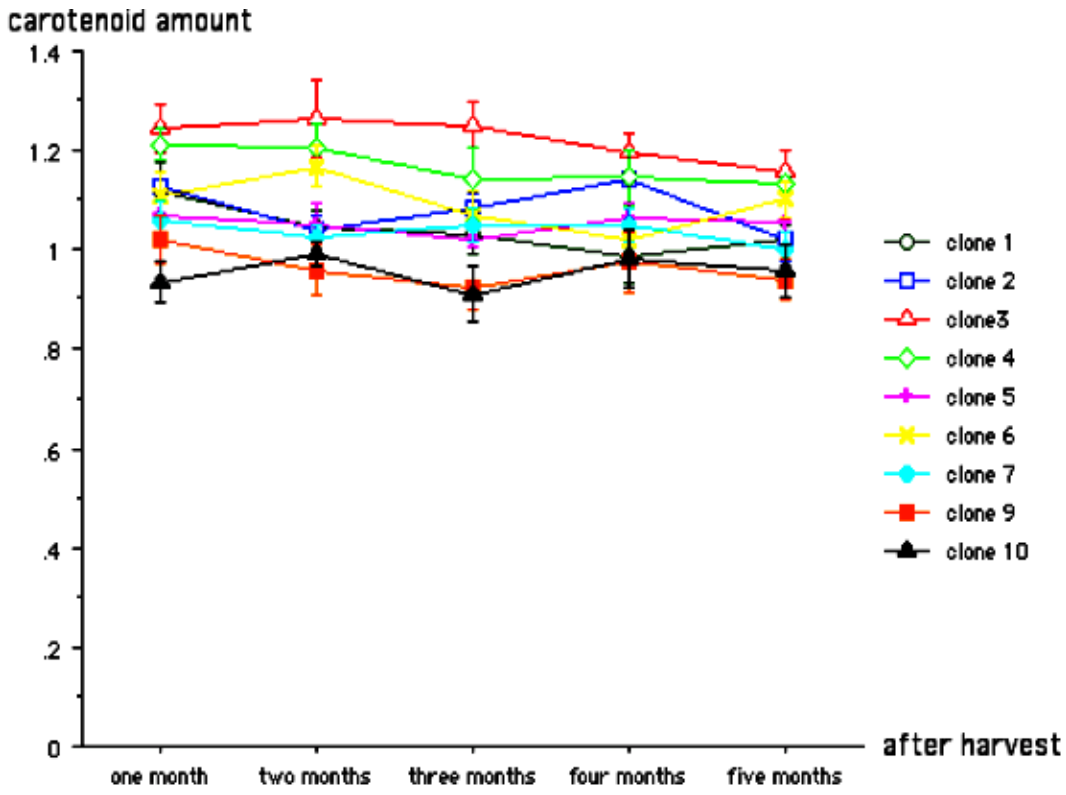

Figure 3: Chart of total carotenoid contents in golden rice grains of nine golden rice lines over five months of storage (ANOVA; $p < 0.05$).

A graph presenting the pooled data of total carotenoid contents for ten transgenic events was showed in figure 4. The effect of storage temperature conditions on stability of total carotenoid contents in grains was observed. Total carotenoid content in grains was only significantly reduced after 5 months of storage (ANOVA; $p<0.05$) when stored at 4°C. Whereas, the demonstrating line for total carotenoid content in grains being stored at room temperature showed small fluctuated. The amount of total carotenoid was significantly reduced after 3 months of storage at room temperature (ANOVA; $p < 0.05$). Therefore, total carotenoid in grains stored at room-temperature seemed to be subjected to degradation earlier than that of grain samples stored at 4°C condition. In other words, carotenoid seemed to be more stable when stored at 4°C than being stored at room temperature. However, the effect of two different storage temperatures on carotenoid content in rice grains was not obviously clear-cut from each other.

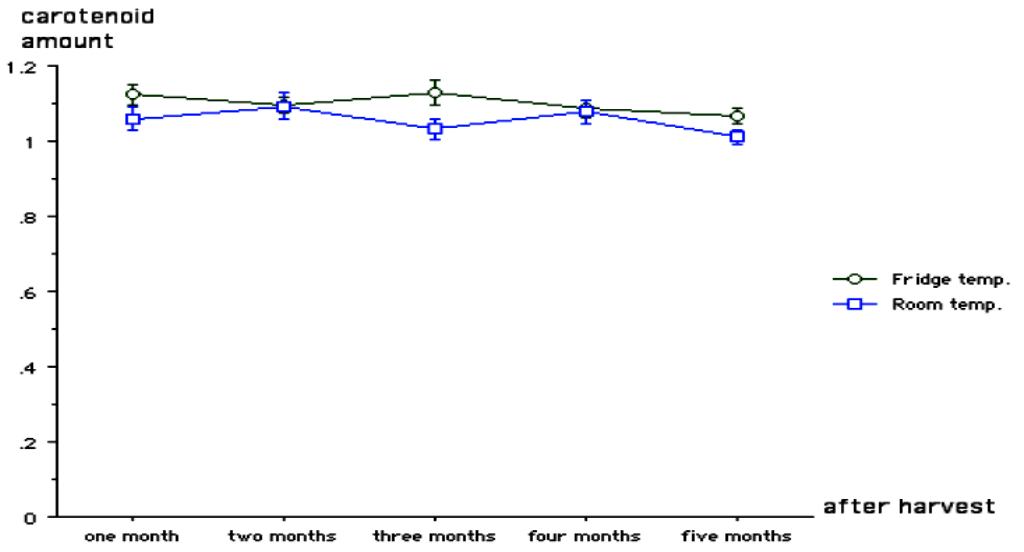

Figure 4: Chart of the pooled data of total carotenoid contents in golden rice grains under the effect of different storage temperature conditions over 5 month-period (ANOVA; $p<0.05$).

Figure 5 showed the graph of the pooled data from the readings at two different storage temperatures for ten transgenic golden lines to evaluate the effect of storage time on level of total carotenoid content in grains. For the first four months of storage, level of carotenoid content in grains showed a little reduction, might be up to 1-2%. However, the reading of total carotenoid content in grains after five-month storage was significantly reduced up to 5 to 10 % as compared to the amount of carotenoid at the first, second, third or fourth readings (ANOVA; $p<0.005$).

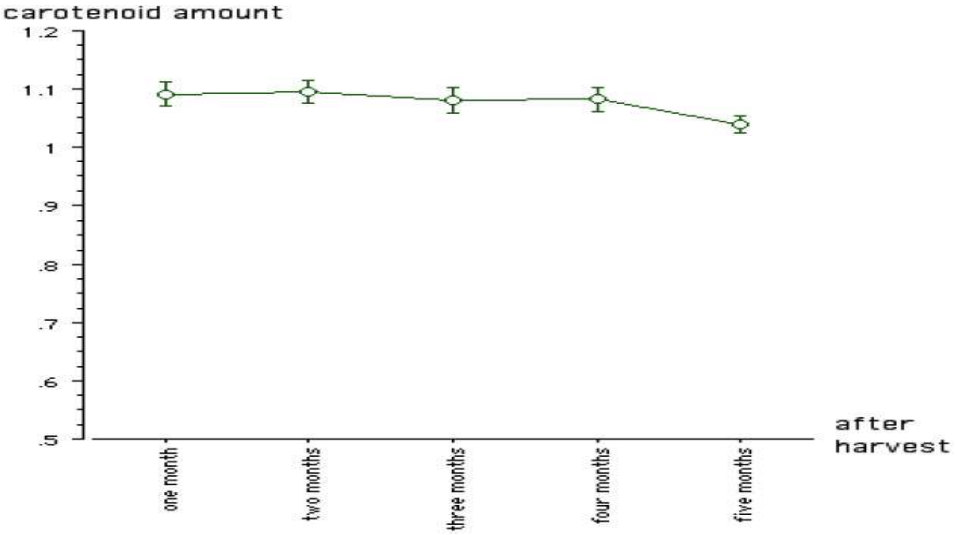

Figure 5: Chart of the pooled data represents for the effect of storage time on total carotenoid contents in golden rice grains over five months of storage (ANOVA; $p < 0.005$).

DISCUSSION

Carotenoids are polyisoprenoid compounds and extremely susceptible to attack by oxygen, free radicals and white light. Thus, a recommended storage protocol has been suggested for preventing the loss of carotenoid is to store samples at low temperatures, under an inert atmosphere, and in the absence of white light. In our experiment, rice endosperms which naturally enclosed in husks were stored in a tigh bag at 4^{o}C or at room temperature conditions. This storage condition (except being stored at room temperature) could inhibit or slow down the loss of carotenoid in rice samples. Besides that, rice grains are often stored in the dry state (its humidity less than 14%). This dehydration prolongs seed health and quality because metabolism, pathogen growth, depletion of food reserves, deleterious by-products are diminished. In this experiment we have shown that the amount of total carotenoid in rice grains seemed to be less changed and less fluctuated when stored at 4^{o}C than that in grains being stored at room temperature condition. The decline of carotenoid level is about 1 to 2% for the first three months and up to 5 to 10% for the last two months over five months of storage. Besides the decline of carotenoid compouds through grain storage, the process of milling, trading and "on-sale" for rice grains might cause a big loss of total carotenoid content. In practice, milled grains are often exposed to light, air and higher humidity conditions for very long time when being on-sale in market. This will futher reduce the amount of beta-carotene in rice grains before consumed. Therefore, all of affecting factors to the amount of grain carotenoid in storage, processing and "on-sale" of rice grains in daily practice need to be investigated.

Hoa et al. (2005) reported the segregation of yellow endosperm vs. white endosperm in T1 seeds of transgenic Taipei 309 golden rice lines following the Mendelian segregation law of 3:1 ratio. They also quantitatively measured major components of carotenoid in rice grains using the HPLC system. The amounts of total carotenoid in their experiment were ranging from 0.2 to 1.0 µg per gram of milled grains of T1 trangenic lines. These amounts were much lower than the previously reported level of 1.6 µg carotenoid per gram of milled grains by Ye et al. (2000) due to the segregation of their T1 seeds. Our result was consistent with the experiments done by Ye et al. (2000) and Hoa et al. (2005). The amount of total carotenoid content in our golden rice grains was varied from 0.83 to 1.37 µg per gram of milled grains in ten transgenic rice lines we analysed. This range did not surpass the amounts obtained by Ye *et.al.* (2000) but was higher than values reported by Hoa et al. (2005) as our transgenic golden rice lines were homozygous in golden-coloured grain trait. As the recommended dietary allowance (RDA) for vitamine A is 1000 retinol equivalents, equal to 6 mg beta-carotene per day for each person. The concentration of total carotenoid in our transgenic golden rice experiment, therefore, is quite far lower than the level required to meet the RDA standard. Hoa et al. (2005) also showed that we are able to select transgenic golden rice lines containing a proportion of beta-carotene in grains reaching up to 50% of 1.6 µg of total carotenoid per gram of mill grains. Our golden rice, if used as a daily staple food, could provide only a fifteen of the RDA (assumed an average consumption of rice is 300-500g per person daily). Pain et al. (2005) reported a breakthrough in developing "Golden Rice two", a second generation of transgenic golden rice that could produce grains containing an increased total carotenoid content in grains up to 23-fold (maximum 37 µg per gram of milled grains) and a preferential accumulation of beta-carotene (up to 80-90% of total carotenoid amount). It means that, with a daily rice intake, we will get sufficient amount of vitamin A for a healthy life. This golden rice should have a big impact on populations of Southeast Asia, where vitamin A deficiency is widespread and rice consumption is high.

In conclusion, our results suggest that: (1) the reading at OD = 475 nm can be used to measure total carotenoid content in golden

rice grains if following our carotenoid extraction method as all wild type readings were almost zero. However, to confirm this reading representing the peak of carotenoid absorbance, an HPLC analysis needs to be done; (2) effect of two different storage temperatures on carotenoid content in rice grains was not obviously clear-cut. However, it is observed that the level of total carotenoid content in grains seemed to be more stable when stored at 4°C than that of rice grains stored at room temperature condition; (3) the decline of carotenoid content in golden rice grains over five months of storage was significant in the last month of storage (about 5 to 10% reduction rate). For the first two or three months of storage, level of total carotenoid content in grains seemed to be more stable or little reduced at non-significant value.

ACKNOWLEDGEMENTS

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Appendix 1

LIST OF GOLDEN TRANSGENIC RICE CLONES USED IN THIS EXPERIMENT

Clone No	Name	Progeny
1	(48-67)-3-3-5-3-2-9-/TP	T6
2	E2-11a F/TP, PN: 2-1-1-23	T4
3	E2-10 F/TP, PN: 3-4-1-30	T4
4	E2-11a F/TP, PN: 2-1-1-17	T4
5	(48-67)-3-3-5-2-1-6/TP	T6
6	(48-67)-3-3-5-2-1-11/TP	T6
7	E2-14b F/D/TP, PN:2-5-3-9	T4
8	E2-14b F/D/TP, PN:2-5-4-23	T4
9	E2-14 F/D/TP, PN:11-2-1-11	T4
10	E2-14 F/D/TP, PN:11-2-1-25	T4

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Ảnh hưởng của tồn trử đến tổng số carotenoid ở hạt lúa vàng (Golden rice)

Hàm lượng tổng số carotenoid trong hạt lúa chuyển gen "Golden rice" được quan sát ở những điều kiện tồn trữ khác nhau, và được ghi nhận ở độ dài sóng OD=475 bằng máy quang phổ kế. Hàm lượng tổng số carotenoid biến động từ 0.81 đến 1.32 µg trong 1 gram hạt gạo được đánh bóng. Ảnh hưởng của hai nhiệt độ tồn trữ đến tổng số carotenoid không khác biệt nhau rõ ràng. Tuy nhiên, khi được tồn trữ ở 4^0C, hàm lượng carotenoid trong hạt dường như ít biến đổi hơn hạt được tồn trữ ở nhiệt độ phòng. Thí nghiệm này cho thấy hàm lượng tổng số carotenoid giảm có ý nghĩa thống kê sau năm tháng tồn trữ, với mức giảm từ 5 đến 10% hàm lượng carotenoid ban đầu. Hàm lượng carotenoid không giảm hoặc ít giảm trong hai tháng đầu tồn trữ ở mức 1-2%.