

# Carotenoid Metabolic Engineering to Functional Rice Crops from *Capsicum* plants

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## Abstract

One of the regulatory mechanisms of the carotenoid biosynthetic pathways has been elucidated by the comparison of gene expression and structure among *Capsicum* varieties that show different ripening colors. Using the carotenogenic genes from Korean red *Capsicum* cloned through this study, we have compared the efficiency of two simultaneous gene expression systems according to the ability to make beta-carotene in rice endosperm. Two bicistronic vectors employing the rice codon-optimized 2A sequence of foot-and-mouth disease virus and the internal ribosome entry site sequence of crucifer-infecting tobamovirus were inserted between two biosynthetic genes encoding *Capsicum* phytoene synthase and *Pantoea* carotene desaturase under a single promoter, respectively. Co-expression of two genes successfully resulted to induce beta-carotene accumulation in both bicistronic systems of 2A and IRES through rice transgene approaches. Currently our research is evolving from beta-carotene to other functional carotenoids like zeaxanthin and astaxanthin by further genetic manipulation of carotenoid biosynthesis. Beyond Golden rice, we hope to develop high nutritional and commercially valuable crops to apply these recent technologies.

## Key Words

Carotenoid, pepper (*Capsicum* sp.), Metabolic engineering, Rice

## Introduction

In plants, carotenoids play indispensable roles in light harvesting, photo-protection and as precursors for abscisic acid (ABA) synthesis. Plants attract insects and other animals to act as pollinators and vehicles of seed dispersion by using stimuli generated by carotenoid pigments, including red, orange and yellow, which are present in fruits and flowers (Bartley and Scolnik, 1995). Carotenoids have also been used for many years as natural colorants in both animal and human foodstuffs, and some of the  $\beta$ -carotenoids are essential components of the human diet as pro-vitamin A. Moreover, some carotenoid components have been shown to have important biological functions as antioxidants and free-radical scavengers, which can reduce the risk of cancer (Hirschberg, 2001). Many of the genes that are involved in carotenoid biosynthesis have now been cloned from several plants including *Arabidopsis*, tomato, pepper, daffodil and marigold (DellaPenna and Pogson, 2006; Ha et al. 1999; Ha et al. 2003). Among these different species of crops, the pepper (*Capsicum* sp. L.) harbors the most evolved carotenoid biosynthetic pathway.

On the other hand, the demand for simultaneous expression of multiple genes has constantly arisen to

manipulate complex metabolic pathway in plants (Halpin et al. 2001; Halpin 2005). Since Golden rice® has showed carotenoids could be accumulated in rice endosperm that is one of plant tissues lacking carotenoid (Ye et al. 2000; Paine et al. 2005), we considered rice endosperm as the best visible system for carotenoid metabolic engineering using the *Capsicum* carotenogenic genes.

## Methods

*Capsicum* varieties were selected from the Genebank of the National Institute of Agricultural Biotechnology (Rural Development Administration, Suwon, Korea) on the basis of their different color types in the fully ripe fruit. Their introduction numbers (IT) are as follows: 158770 (*C. baccatum* var. *pendulum*, LR2), 158773 (*C. chinense*, R2), 158782 (*C. annuum*, W), 158806 (*C. baccatum*, LR1), 163499 (*C. annuum*, DR), 164918 (*C. chinense*, Y3), 191655 (*C. chinense*, PYO), 203499 (*C. chinense*, O), 709424 (*C. pubescens*, R1), 800065 (*C. chinense*, Y2) and 800070 (*C. baccatum*, Y1). We also included a commercial cultivar in these analyses (*C. annuum* cv. Nockwang, R3), which was purchased from HungNong Seeds Company (Seoul, Korea). Pepper fruits of all 12-pepper varieties were harvested at fully expanded stages where differences in their respective colors during ripening were evident. At this time, the fruit colors were also visually recorded as shown in Figures 1. Their carotenoid pigments were extracted to quantify by HPLC and total RNAs were also purified from the same fruit samples used in the HPLC analysis for northern blot analysis.

Rice embryos (*Oryza sativa* cv. Nacdong) were used to produce transgenic plants by co-cultivation with *A. tumefaciens* strain LBA 4404 including super binary vectors, respectively. Total RNAs and proteins of transgenic rice plants were extracted from dry seeds of T3 generation for northern and western blot analyses.

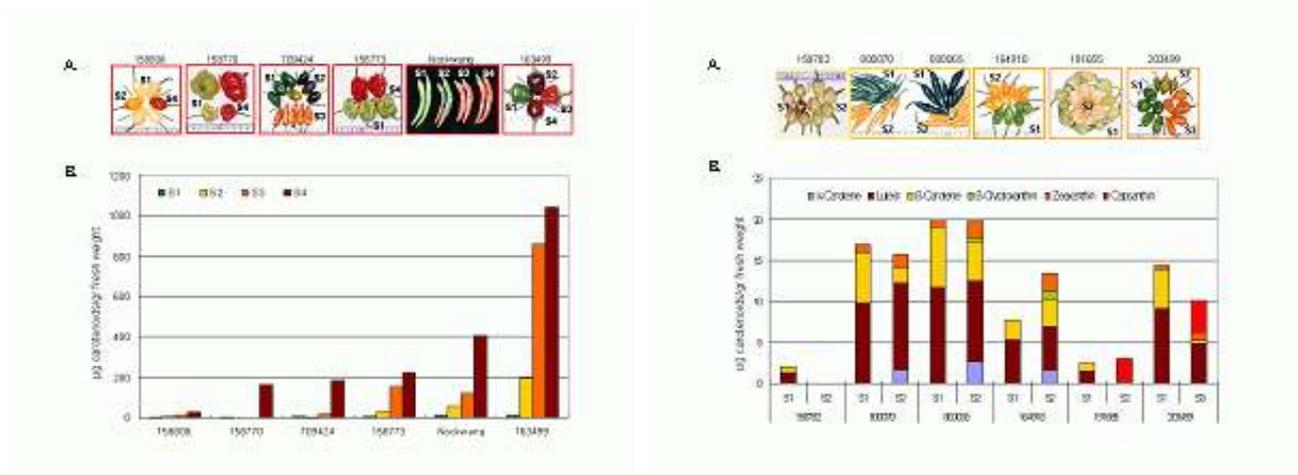
## Results

Ripe pepper (*Capsicum* sp. L.) fruits can display a range of colors, from white to deep red. To better understand the regulatory mechanisms of the carotenoid biosynthetic pathways that underlie these ripening colors, *Capsicum* varieties that show seven different fully ripe color types were analyzed. The levels and composition of the carotenoid accumulation in these samples at different stages of ripening were measured, and the resulting data were analyzed in conjunction with the expression patterns of the carotenoid biosynthetic genes (Ha et al. 2007). We find that red peppers accumulate increasing levels of total carotenoids during ripening, whereas non-red peppers accumulate lower levels of total carotenoids of varying composition (Fig. 1). The expression levels of the phytoene synthase, phytoene desaturase, and capsanthin-capsorubin synthase (*Ccs*) genes are high in peppers with high levels of total carotenoid, whereas one or two of these genes are not expressed in peppers with lower levels of total carotenoid. Surprisingly, we found that the *Ccs* gene is present in two *Capsicum* varieties whose ripe-color is yellow. This gene has never previously been shown to be present in yellow peppers. Sequence analyses of the *Ccs* gene further revealed two structural mutations in yellow peppers that may result in either a premature stop-codon or a frame-shift. Taken together with the fact that the *Ccs* transcript is not detectable in the yellow peppers, our current results suggest that nonsense-mediated transcriptional gene silencing of *Ccs* and not the deletion of this gene is responsible for yellow ripening in *Capsicum* (Ha et al. 2007).

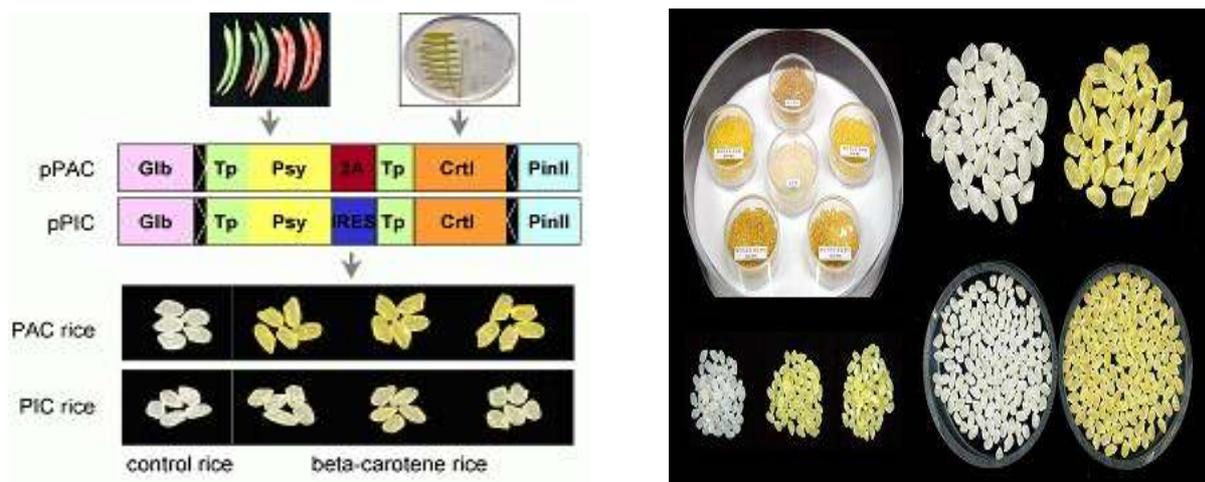
Through rice transgene approaches, two bicistronic vectors employing the rice codon-optimized 2A sequence of foot-and-mouth disease virus (FMDV) and the internal ribosome entry site (IRES) sequence of crucifer-infecting tobamovirus (CrTMV) were compared by insertion between two biosynthetic genes encoding *Capsicum* phytoene synthase (PSY) and *Pantoea* carotene desaturase (CrtI) under a single promoter, respectively. We examined the efficiency of two systems available to coordinately express multiple metabolic genes on transcriptional and translational levels. As results, the content of beta-carotene accumulated in rice endosperm by 2A sequence was five times greater than that by CrTMV-IRES sequence.

(L)

(R)



**Figure 1. Photographs (A) and the Total Carotenoid Contents (B) in Red (L) and Non-red (R) *Capsicum* Varieties during Ripening.**



**Figure 2. Beta-carotene Accumulation by Two Bicistronic Gene Expression in Rice Grain.**

**Conclusion**

The effort through *Capsicum* study has furthered our understanding of carotenoid biosynthesis. In particular,

the data confirm and extend previous studies on the function of capsanthin-capsorubin synthase (*Ccs*). It is shown that inactivation of the *Ccs* gene in yellow pepper is not mediated by deletion of the gene but rather by transcriptional gene silencing. Moreover, success of two bicistronic systems to coordinately express multiple genes in plants will help us to increase the total carotenoid contents of crop plants, direct the carotenoid biosynthetic machinery towards other useful carotenoids, and produce a new array of carotenoids by further metabolizing the new precursors in the near future.

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