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Full Length Research Paper

Expression of phytoene synthase (*psy*) gene and its relation with accumulation of carotenoids in tea [*Camellia sinensis* (L) O Kuntze]

Borthakur D, Lu JL, Chen H, Lin C, Du YY and Liang YR*

Zhejiang University Tea Research Institute, 268 Kaixuan Road, Hangzhou 310029, China.

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Carotenoids are important determinant of tea quality as many of the quality related flavour volatiles are produced through the degradation of carotenoids during tea processing. Variation in levels of carotenoids including neoxanthin, violoxanthin, xanthophylls and β-carotene in early and late period of spring, summer and autumn seasons in two tea cultivars were investigated. The relationship of phytoene synthase (*psy*) gene expression to the accumulation of carotenoids in tea was studied. The results showed that carotenoids level in late period of a plucking season was consistently higher than its early period though carotenoids accumulation patterns of the two tea cultivars over different plucking periods was different. The carotenoids accumulation showed a strong seasonal dependence for the expression of *psy* gene and psy gene transcript abundance is considered to be an important indicator for screening tea cultivars with high level of carotenoids.

Key words: Carotenoids, phytoene synthase, gene expression, seasonal variation, tea quality, volatile compounds.

INTRODUCTION

Carotenoids, a group of isoprenoid-derived pigments, are produced by all photosynthetic and many non-photosynthetic organism. The importance of carotenoids in photosynthetic tissue is immense and plant lacking carotenoids can not survive in the light because carotenoids function both in the acquisition of light energy and in the protection of photosynthetic apparatus against excessive light damage (Demmig-Adams et al., 1996). Carotenoids are also exploited as coloring agents in flowers and fruits to attract pollinators and agents involved in seed dispersal (Cunningham and Gantt, 1998). Besides, carotenoids biosynthetic pathway provides intermediates for the biosynthesis of the plant growth regulator abscisic acid (Neill et al., 1986) and they are precursor of vitamin A for human and animal diet (DellaPenna, 1999; Hirschberg, 1999). Evidence for the requirement of novel carotenoid derived signaling compounds that regulate aspects of plant development, in particular apical dominance and branching, is accumulating in recent years (DellaPenna and Pogson, 2006).

Carotenoid biosynthetic pathway takes place within the plastid, and the first committed step is the head to head condensation of two geranylgeranyl diphosphate (GGDP) molecules to produce phytoene (a colorless carotenoid) which was catalyzed by the enzyme phytoene synthase (psy) (Cunningham and Gantt, 1998). The contribution of transcript abundance of *psy* to the carotenoids accumulation was studied in many plant species. The transcript level of *psy* was observed to increase with the accumulation of carotenoids in tomato fruit (Giuliano et al., 1993), citrus (Ikoma et al., 2001) and pepper fruit (Bouvier et al., 1994). However, in daffodil flower, the expression of *psy* during flower development remained constant or even decreases (Schledz et al., 1996). In marigold, Moehs et al. (2001) reported that expression and/or stability of *psy* transcripts is the key requirement for the accumulation of carotenoids. In contrast, Del Villar-Martinez et al. (2005) reported in three different varieties of marigold that induction of *psy* transcript was not related to pigment accumulation. There was tissue specific psy gene in some crops. Two differentially regul-

*Correspondence author. E-mail: yrliang@zju.edu.cn. Fax: +86 571 86971704.

ated *psy* genes had been reported in tomato, i.e. *psy-1* to be responsible for carotenoid biosynthesis in fruit and *psy-2* for foliar carotenoids (Fraser et al., 1994). However, in citrus, no such isoforms were detected (Kim et al., 2001).

Tea (*Camellia sinensis*) is a perennial woody plant and its young shoot is used for the production of a non-alcoholic beverage. Because of the beneficial health effects including antioxidant and antibacterial functions (Carori et al., 2007; Oyejide and Olushola, 2005; Mbata, 2007; Esimone et al., 2006), tea is very popular as a health drink and is the most consumed liquor next only to water in the world recent years. Tea germplasm are broadly categorized into three groups: China type (*Camellia sinensis*) with small leaf, Assam type (*Camellia assamica* Masters) with large leaf and Cambodia type (*Camellia assamica* Masters sub sp. *Lasiocalyx*) with intermediate leaf size (Bezbaruah, 1975). Assam type is suitable for production of black tea (fermented) whereas China type is used mainly for production of green (non-fermented) and oolong tea (semi-fermented) tea.

Flavor of the finished product is an important determinant of tea quality. Many of the flavor compounds that determine tea sensory quality are compounds formed through degradation of carotenoids during processing. Wide variation for carotenoids content was reported in the three types of tea plants (Ravichandran, 2002) and flavorsome tea is generally produced from cultivars with high carotenoids content (Wickremasinghe, 1974). Carotenoids content is considered to be an important quality parameter (Hazarika and Mahanta, 1984) and information regarding regulation of the carotenoids biosynthetic genes, their contribution towards carotenoids accumulation, gene expression pattern are needed to breed variety having high carotenoids. However, literature on these aspects of tea is lacking.

In the present work, seasonal variation in levels of predominant carotenoids including xanthophyll, violoxanthin, neoxanthin and β-carotene in two different tea cultivars and the relationship of *psy* gene expression to the accumulation of the carotenoids were examined.

MATERIAL AND METHODS

Plant materials

Two tea cultivars, Longjing-43 (LJ-43) and Yulan (YL), were grown under identical crop management practices in the Experimental Tea Farm of Zhejiang University (Hangzhou, China). LJ–43 is a green tea cultivar with small China type leaf and Yulan is a cultivar with broad Assam type leaf used for production of black tea. To eliminate the differences in leaf development stage of various plucking seasons, second leaf (10 - 15 g) from tea shoots consisting of two leaves and a bud was harvested from each cultivar in the early and the late period of three plucking seasons i.e. spring, summer and autumn. Early period was defined as the period when all the shoots were in actively growing stage, whilst in the late period; about 50% of the shoots were entered into the dormant stage. Harvested tea shoots were kept at - 80ºC until use.

Extraction and HPLC analysis of pigments

500 mg of leaf was crushed into fine paste with 5 ml of cool acetone, centrifuged at 4,300 × g for 12 min and the supernatant is used for HPLC analysis. Chromatography was carried out using a 2010A HPLC system (Shimadzu, Kyoto, Japan). Data were collected using the ClassVP software supplied with the system. Throughout chromatography, the eluate was monitored continuously by UV–visible detector at 450 nm. Column temperature was maintained at 35°C . A reverse phase C_{18} Synergi 4U Fusion-RP 804 (150 x 4.6 nm) coupled to a guard column (Fusion-RP 4 × 30 mm, Phenomenex Co., CA,USA) was used. Two mobile phases 'A' and 'B' were used as a gradient at a flow rate of 1.0 ml min^{-1}. Mobile phase 'A' was a mixture of acotonitrile / acetic acid / water (3/5/96.5, v/v/v). Mobile phase 'B' was acetonitrile /methanol / chloroform (75/20/5, v/v/v). For separation of the individual carotenoid, gradient elution program was applied as follows: phase 'B' linearly increasing from 80 to 100% during the early 20 min and then holding at 100% 'B' for the following 15 min. Identification and quantification of neoxanthin, vialoxanthin, xanthophyll, and β-carotene was made by co-chromatography with standards (Sigma Chemicals, St Louis, MO, USA) of known amount. Total carotenoids was calculated by summing up the detected individual carotenoids.

Determination of *psy* gene expression

Total RNAs were extracted from the sampled leaf using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the product instruction manual. Quality of the extracted RNAs was checked by measuring the absorbance at 260 and 280 nm on a spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, UK) and RNAs with ratio of OD_{260}/OD_{280} ranging from 1.8 to 2.0 were used for cDNA synthesis. The first strand cDNA was synthesized from 2.5 µg of total RNA and 0.5 µg of oligo (dT) primer using a first strand cDNA synthesis kit (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China) as described by the manufacturer. The synthesized cDNA was stored at -20ºC for gene expression study.

Primers for *psy* gene were designed from the *psy* gene of *Citrus sinensis* (GenBank access No.DQ235260.1) and and those for *β-actin* gene were designed from *Populas trichocarba* (GenBank access No.EF418792) using the Lasergene Primer Select (DNAStar, Madison WI, USA) software. The primers for *psy* were: forward primer (5'-GATGGGCCTAATGCTTCACAC-3') and reverse primer (5'-CTTGCCCTCTTAATTTGGTTCTTC-3') and that for *β-actin* were: forward primer(5'-AAAGCAAACAGAGAAAAGATGACC-3') and reverse primer (5'-AGCACCAATAGTAATGACCTGACC-3'). The predicted length of the fragments to be obtained was 434 bp for *psy* and 419 bp for *β-actin*. PCRs were performed on a PTC-221 Dyad Disciple Cycler (MJ Reserch, Waltham, Massachusetts, USA) according to the following conditions: preheating at 95ºC for 5 min, followed by 45 cycles with 15 s melting step at 95ºC , 50 s annealing step at 55.4º (for *psy*) or 54ºC (for *β-actin*) and extension step at 72ºC for 1.2 min; and finally one more cycle at 72ºC for 5 min for strand extension. The experiments were repeated twice. The PCR products were fractionated on 1.5% (w/v) agarose gels. The expected bands were purified, cloned into pMD18-T Vector (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China) and sequenced to verify the identity of the PCR amplicons. The sequencing was carried out according to the dideoxynucleotide chain termination method using an ABI Prism Sequencer (Applied Biosysytem, Foster City, USA). Sequenece comparison was caried out using the BLAST programme (Altschul et al., 1997) and the obtained nuclotide sequence of *psy* was submitted to the NCBI (Accession no: EF 545005).

To compare the mRNA expression levels of the *psy* gene of the

Table 1. Carotenoids content in different plucking periods (μg g^{-1}, fresh weight)*.

Cultivar	Season	Neoxanthin	Violoxanthin	Xanthophull	β-Carotene	Total
YL	Early spring	5.00c	5.07c	161.45c	30.33c	201.85c
YL	Late spring	8.20a	11.09a	192.15a	39.57a	251.01a
YL	Mean	6.60	8.08	176.8	34.95	226.43
YL	Early summer	4.15d	6.64b	131.53f	19.16 d	161.48f
YL	Late summer	6.63b	7.66b	142.33e	21.16d	177.77e
YL	Mean	5.39	7.15	136.93	20.16	169.63
YL	Early autumn	3.47d	4.39c	149.95d	29.89c	188.91d
YL	Late autumn	5.21c	5.33c	165.55b	33.97b	210.06b
YL	Mean	4.34	4.86	157.75	31.93	199.49
LJ-43	Early spring	3.99d	5.56c	162.11f	31.35f	203.01f
LJ-43	Late spring	5.45d	6.42c	183.36e	36.32e	231.54e
LJ-43	Mean	4.72	5.99	172.73	33.83	217.28
LJ-43	Early summer	13.05b	16.05b	366.01b	111.89b	506.99b
LJ-43	Late summer	18.95a	21.46a	483.08a	124.17a	647.65a
LJ-43	Mean	16.00	18.75	424.54	118.03	577.32
LJ-43	Early autumn	5.96d	7.06c	237.17d	39.62d	289.80d
LJ-43	Late autumn	7.19c	7.42c	259.51c	42.18c	316.30c
LJ-43	Mean	6.575	7.2375	248.34	40.9	303.05

*Statistical analysis for the two cultivars was made separately.
Values followed by a different lower-case letter in the same horizontal row were statistically different at $P = 0.05$, n=2.

two cultivars in different plucking periods, the PCR products on the agarose gel (1.5%, w/v) were stained by ethidium bromide and the intensity of each band was scanned and recorded by a Gel Documentation System (JeDa Technologies, Nanjing, China).

Data analysis and statistics

The observations were made two replications and the mean value of the two replications are presented. Data were analysed using the software SAS V8.01 (SAS Institute Inc., NC, USA).

RESULTS AND DISCUSSION

Cultivar and seasonal variation in level of carotenoids

Table 1 showed cultivar and seasonal variation in levels of predominant carotenoids. Levels of single component of carotenoids were differed greatly and xanthophyll was the most abundant component among the detected carotenoids, followed by β-carotene and violoxanthin. Neoxanthin level was the lowest. Accumulation patterns of carotenoids were different between cultivars. Total carotenoids of LJ-43 (Longjing-43) were higher than that of YL (Yulan) in every season and every plucking, especially in the summer pluckings. Seasonal variation in level of total carotenoids depended on cultivars. On seasonal average, YL had the highest level of total carotenoids in the spring, and the lowest in summer, with autumn in between. LJ-43 had the highest level of total carotenoids in summer and the lowest in spring, with autumn in between (Table 1). Between pluckings, YL had the highest total carotenoids in late spring, followed by late autumn and early spring. Total carotenoids level in YL was the lowest in early summer. LJ - 43 had the highest total carotenoids level in late summer, with early summer the next. It was the lowest in the early spring plucking. In the other pluckings, it ranged from 203.01 μg g^{-1} (fresh weight) to 316.30 μg g^{-1}. It was interesting that concentrations of single component of carotenoids and total carotenoids were always higher in late plucking than in early plucking of the same season. It showed a same trend in the two cultivars (Table 1). Carotenoids are important precusors of many tea flavour volatiles (Ravichandran, 2002). The present study suggests that late plucking for a same tea cultivar in each production season has a higher potential for processing flavoursome tea than its early plucking though the accumulation patterns of various tea cultivars were different.

It is known that oolong tea made in China has stronger aroma than green tea and black tea. It is processed using dormant shoots plucked in the late periods of every plucking season. It was confirmed that a lot of aromatic volatiles were degraded products of carotenoids. This may be the reason that oolong tea is harvested later than green tea and black tea every season.

Relationship between expression of psy gene and carotenoids accumulation

Total RNAs of leaf from the six pluckings of two cultivars

Table 2. Band intensity of *psy* in various pluckings.

Cultivar	Season	Band intensity
YL	Early Spring	155.05
YL	Late Spring	190.35
YL	Early Summer	81.17
YL	Late Summer	115.63
YL	Early Autumn	150.36
YL	Late Autumn	155.26
LJ-43	Early Spring	106.29
LJ-43	Late Spring	148.14
LJ-43	Early Summer	171.41
LJ-43	Late Summer	197.18
LJ-43	Early Autumn	123.51
LJ-43	Late Autumn	205.63

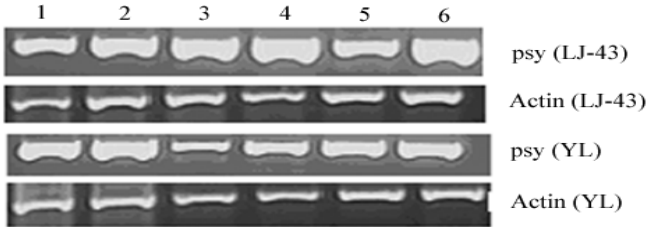

Figure 1. Expression profiles of psy gene in various pluckings of two tea cultivars. 1. Early spring; 2. Late spring; 3. Early summer; 4. Late summer; 5. Early autumn; 6. Late autumn.

were extracted and fragment of *psy* gene was cloned. The sequencing result showed that the obtained fragment had high similarity to the other plant sources; 97% to *Lycopersicon esculentum* (NCBI Accession no: M84744.1), 95% to *Daucus carota* (NCBI Accession no: DQ192187.1), 94% to *Prunus mume* (NCBI Accession no: AB 253628). It suggests that the obtained fragment is from *psy* gene of tea plant.

The expression profile of the *psy* gene in different pluckings of the two cultivars is presented in Figure 1. The *Psy* expression patterns of cultivars YL and LJ-43 showed a similar trend with their accumulations of carotenoids, respectively (Tables 1 - 2 and Figure 1). For cultivar LJ-43, high expression of *psy* gene was observed in late summer, early summer and late autumn and low expression was in early spring, with early autumn and late spring in between. Although the highest expression was observed in the late autumn, a concomitant increase in carotenoids was not found. This might be due to the climate conditions that were not favourable for the expression of its downstream genes of the pathway. The expression in late period of every season was higher than that of early period. This was similar to the changes in level of total carotenoids. In cultivar YL, the highest *psy* expression was in spring season and the lowest in summer season, with autumn in between. This showed a same tendency with seasonal average levels of total carotenoids (Table 1). These confirmed that the accumulation of carotenoids was regulated by the expression strength of *psy* gene in tea plant.

The differentiation of *psy* gene expression patterns of different tea cultivar might be owing to their different responses to environment conditions. An albino mutant of tea plant with high concentration of amino acids and low carotenoids has white leaf when environmental temperature is below 20℃ (Du et al., 2006). Carotenoids content in tea was influenced by climatic factors (Matthews and Stephens, 1998). Hazarika and Mahanta (1984) showed that carotenoids content was maximum in spring and lowest in summer in North East India. Expression of carotenogenic genes was influenced significantly by environmental factors and white-light illumination stimulated expression of xanthophyll biosynthetic genes including β-carotene hydroxylase (*bhy*) (Woitch and Römer, 2003). Such kind of seasonal dependence of gene expression was also reported in poplar leaves where expression rates of isoprenoid biosynthesis–related genes were highly variable over the growing season, with temperature and light as the most obvious factor controlling the transcript level in fully developed poplar leaves (Mayrhofer et al., 2005). Light illumination and temperature conditions might be the reason that high concentrations of carotenoids were accumulated in the late period of a season.

The present study showed that expression of *psy* gene of tea cultivars was closely correlated to accumulation of carotenoids which are precursors of tea flavour volatiles. It suggests that the expression strength of *psy* gene can be used as an indicator for screening quality tea cultivars.

The carotenoids are products of the downstream genes of carotenoid pathway and the psy gene regulates the first committed step of the pathway (Cunningham and Gantt, 1998). The study on these downstream genes will provide more insights in the role of transcripts level of different genes on overall carotenoids accumulation and

will be very helpful in screening germplasm for high carotenoids content in an early stage of tea breeding.

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