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Isolation and expression of the genes encoding the early carotenoid biosynthetic enzymes in the fruit peel of pummelo (*Citrus grandis* cv. Melomas) during maturation

[Pemencilan dan ekspresi gen yang mengekodkan enzim pada awal tapak jalan biosintesis karotenoid di dalam kulit limau bali (*Citrus grandis* cv. Melomas) semasa kematangan]

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Key words*:* carotenoid accumulation, cDNA cloning, *phytoene synthase*, *phytoene desaturase*, *lycopene beta-cyclase*, mRNA expression, RT-PCR, RTq-PCR

Abstract

Three different partial cDNA clones encoding the early carotenoid biosynthetic enzymes, *pummelo phytoene synthase* (*PumPSY*), *pummelo phytoene desaturase (PumPDS*) and *pummelo lycopene-beta-cyclase* (*PumLYCb*) were isolated from the peel (flavedo) of the local (*Citrus grandis* cv. Melomas) citrus fruit. Comparison of the deduced partial amino acid sequences of all three genes showed more than 90% identity with the Satsuma mandarin (*Citrus unshiu* Marc.), *Citrus* x *Paradisi* and *Citrus sinensis*.

Expression analysis revealed a high level of the *PumPSY* transcript in the peel at the early stages of fruit development which declined gradually towards the third month of fruit development and then accumulated again during fruit maturation. In contrast, the *PumLYCb* transcript was present in lower levels than *PumPSY* in the peel at the early developmental stages and then increased slightly towards fruit maturation. On the other hand, the *PumPDS* transcript remained in very low amounts throughout the developing stages compared to *PumPSY* and *PumLYCb*. The expression patterns indicated non-coordinated regulation of the genes and the fluctuations were not in accordance with carotenoid accumulation and chlorophyll disappearance that leads to the peel colour change from green to orange as observed in the flavedos of Satsuma mandarin and Valencia oranges.

Introduction

The majority of citrus carotenoid studies have involved peel carotenoids for two reasons. Firstly, these pigments are responsible for the desirable colour of the fruit, and secondly, peel is the most concentrated source of these pigments in the fruit. Carotenoids are synthesized and accumulated in plastids (von Lintig et al. 1997) and are involved in many functions related to accessory pigments in chloroplasts of photosynthetic tissues, photoreceptors and precursors to the hormone, abscisic acid (ABA) (Li et al. 1996). In addition, some carotenoids serve as precursors for vitamin A, which is essential to animal diet, and as antioxidants, which play a role in reducing the risk of certain forms of cancer (Olson 1989). It has also been demonstrated that β-cryptoxanthin and lutein have potential

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anti-tumour properties superior to the well known anti-tumour promoter β-carotene (Tsushima et al. 1995).

Carotenoids are derived from the isoprenoid pathway. In the initial stages of carotenoid biosynthesis pathway in plants, the key enzyme *phytoene synthase (PSY)*, reported to be a peripheral plastid membrane protein, can be considered as one of the important genes for the regulation of the characteristic carotenoid accumulation. The first step is catalyzed by this enzyme which converts two molecules of geranylgeranyl pyrophosphate (GGPP) (C_{20}) into the symmetrical 40 carbon phytoene, (the first C_{40} carotenoid) via the intermediate prephytoene pyrophosphate (PPPP) (Daito et al. 1975; Cunningham and Gantt 1998). Subsequently, this colourless compound phytoene, undergoes a series of four sequential desaturation steps to form first, phytofluene and then, in turn, converted into yellow (ζ-carotene), orange (neurosporene) and red (lycopene) carotenoids by introducing the conjugated double bonds (Daito et al. 1975; Bartley and Scolnik 1995; Cunningham and Gantt 1998). In plants and algae, these steps are catalyzed by two enzymes, *phytoene desaturase* (*PDS*) and ζ-*carotene desaturase* (*ZDS*).

Cyclization reaction of *lycopene cyclase* (*LYC*) enzyme into *β*- and/or *ε-cyclases* convert lycopene to either δ- or γ- primary carotenoids. When present together, *β*- and *ε-cyclases* convert ζ-carotene to α-carotene which is then hydroxylated to lutein (yellow pigment) by *α-carotene hydroxylase* (Cunningham et al. 1996). On the other hand, the presence of *lycopene β-cyclase (LYCb)* alone converts γ-carotene to β-carotene by catalyzing the formation of two beta-rings at each end of the linear carotene, which is then hydroxylated to β-cryptoxanthin and zeaxanthin (orange pigments) by *β-carotene hydroxylase* as in the case of the Satsuma mandarin (*Citrus unshiu* Marc.) peel (Ikoma et al. 2001). These xanthophylls or oxygenated carotenoids are important constituents of the photosynthetic membrane. The desaturation and cyclization reactions occur within plastids and are catalyzed by integral membrane enzymes (Bramley 1985).

Maturation of most citrus fruits which accumulate a large amount of carotenoids (Stewart 1977) leads to the pigmentation of the colour orange during ripening as observed in the Satsuma mandarin. This rapid accumulation of carotenoids, particularly β-cryptoxanthin and zeaxanthin, takes place concomittantly with a decrease of chlorophyll (Daito et al. 1975). However, the colour of the local Malaysian pummelo (*Citrus grandis* cv. Melomas) peel remained green until maturity and gradually changed to yellow as shown in *Plate 1*.

From the previous study using Reverse-Phase High Performance Liquid Chromatography (RP-HPLC), lutein is the major carotenoid present in the peel throughout all stages of fruit development (Kashim et al. 2005). However, molecular changes that underlie carotenoid biosynthesis in citrus are poorly understood despite the biochemical and pharmacological importance of citrus. Therefore, as a first step in a comprehensive analysis of carotenoid gene regulation in this citrus peel, the two partial clones, *pummelo phytoene synthase (PumPSY)* and *pummelo phytoene desaturase (PumPDS)* and one recently completed *pummelo lycopene-beta-*

Plate 1. Different stages of fruit development in pummelo ***Citrus grandis*** *cv. Melomas*

cyclase (PumLYCb) cDNA clone were isolated and their expression during fruit development were analyzed. These preliminary findings would make it possible to suggest a pathway for carotenoid biosynthesis in the peel of this local citrus fruit and an explanation for the lack of colouration from green to orange of the pummelo peel during the ripening stages.

Materials and methods

Pummelo (*C. grandis* cv. Melomas) fruits cultivated at MARDI Station, Jelebu (Negeri Sembilan, Malaysia) were collected periodically every month for 5 months consecutively during growth and maturation while Sunkist oranges (*Citrus sinensis* Valencia 4014) were bought from a local market. Flavedo *(*peel) was separated from other parts of the fruit, weighed, immediately frozen in liquid nitrogen and stored at –80 °C until further use.

Total RNA isolation

Total RNA was extracted from the pummelo (cv. Melomas) peel at different stages of fruit development (1, 2, 3, 4 and 5 months after flowering) and from the mature Sunkist Valencia peel using the method described by Matsumura et al. (1999).

Detection of PumPSY, PumPDS and PumLYCb partial genes and isolation of full-length PumLYCb

Reverse-transcription and polymerase chain reaction (RT-PCR) was used to amplify the partial *PSY*, *PDS* and *LYCb* genes from total RNA of Sunkist peel and these were then used as positive controls to amplify the same from the pummelo peel. First-strand complementary DNA (cDNA) was synthesized from 5 µg total RNA using the RT kit from Promega (USA) and oligo $dT_{(15)}$ primer. PCR was performed on the first strand cDNA using the following cycle conditions: 10 min at 95 °C followed by 30 cycles of 1 min at 94 °C, 45 s at 50 °C for *PSY* and *PDS* and 46 °C for *LYCb*, and 45 s at 72 °C using the MJ DNA engine (PTC200, USA). The degenerate sense and antisense primers used for isolation of the partial genes were designed and synthesized based on homologous sequence regions of the *PSY* gene in *Arabidopsis thaliana* (L25812), *Lycopersicon esculentum* (M84744), *Capsicum annuum* (X68017), *Citrus* x *Paradisi* (AF152892) and *Citrus unshiu* (AB037975), *PDS* gene in *Glycine max* (M64704), *Zea mays* (U37285), *Capsicum annuum* (X68058), *Oryza sativa* (AF049356), *Lycopersicon esculentum* (X59948) and *Arabidopsis thaliana* (L16237) and *LYCb* gene in *Arabidopsis thaliana* (U50739), *Lycopersicon esculentum* (X86452), *Capsicum annuum* (X86221) and *Adonis palaestina* (AF321534) from the genebank. The full length *PumLYCb* was isolated using the 5' RACE kit.

Cloning, sequence and expression analysis

The amplified fragments were cloned into the pCR2.1-TOPO vector with a TA cloning system (Invitrogen, U*S*A) and their identity confirmed by DNA sequencing using the 377 ABI ((Perkin-Elmer Applied Biosystems, USA). Gene-specific forward and reverse nested primers were designed for reverse transcriptase-polymerase chain reaction (RT-PCR) expression studies for the *PumPSY* and *PumLYCb* genes in the peel at different stages of fruit development. The same forward and reverse primers were used for the *PumPDS* RT-PCR expression studies. The RT-PCR expression profiles obtained for *PumPSY* and *PumLYCb* genes were validated by relative real-time quantitative-polymerase chain reaction (RTq-PCR) using the Opticon I DNA Engine (MJ Research, USA) and the DyNAmo SYBR Green Kit (Finnzymes, Finland).

Results and discussion

Detection, isolation and sequence analysis of partial PumPSY, PumPDS, PumLYCb and full-length PumLYCb

The sizes of the partial cDNA fragments detected for the *PumPSY*, *PumPDS* and *PumLYCb* genes were 475 bp, 794 bp and

792 bp, respectively. The deduced partial amino acid sequences of *PumPSY* and *PumPDS* showed more than 90% identity with *PSY* of *Citrus* x *Paradisi* (AF152892), *C. unshiu* Marc. (AF220218) and *C. sinensis* (AY204550) *(Figure 1)* and *PDS* of *Citrus* x *Paradisi* (AF364515), *C. unshiu* Marc. (AB046992) and *C. sinensis* (AB114657) *(Figure 2)*. The isolated nucleotide sequence of *PumLYCb* cDNA clone (1678 bp) contains the complete protein coding sequence of 505 amino acid residues *(Figure 3)* with two possible cleavage sites (55th or 56th nucleotide sequence), a putative signal peptide of 9 amino acid residues at the N terminus and a potential NAD-binding site domain at position 80–442 of the nucleotide sequence. The location of the cleavage sites at these positions would generate a mature protein of molecular mass approximately 56.4 kDa with a pI of 7.20. The secondary structure predicted for this clone is shown in *Figure 4* and the deduced amino acid sequence showed more than 94% identity with *LYCb* of *C. unshiu* (AY166796), *Citrus* x *Paradisi* (AF152246) and *C. sinensis* (AF240787) *(Figure 5)*. The partial *PumPSY* and recently completed *PumLYCb* cDNA clones have been deposited in the DNA database under the accession numbers AY184808 and AY217103, respectively. However, only the completed *PumLYCb* cDNA clone has been characterized.

Expression analysis of PumPSY, PumPDS and PumLYCb in the peel during fruit development

Expression analysis indicated that the *PumPSY* gene expression appeared to be much stronger in the pummelo peel throughout the developmental stages compared with *PumLYCb* and *PumPDS*. The transcript was detected at a high level in the 1-month peel, decreased slightly toward the third month of fruit development before increasing again during fruit maturation *(Plate 2)*. In contrast, the transcript corresponding to the *PumLYCb* mRNA was

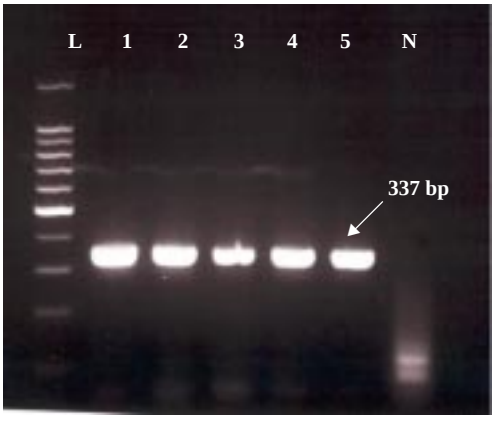

Plate 2. RT-PCR expression of PumPSY in the peel at 1, 2, 3, 4 and 5 months of fruit development, N : negative control & L : 100 bp ladder

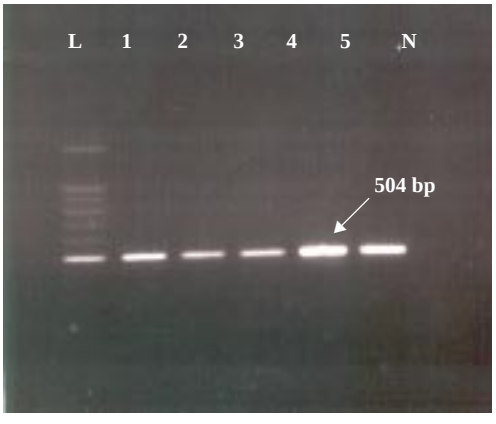

Plate 3. RT-PCR expression of PumLYCb in the peel at 1, 2, 3, 4 and 5 months of fruit development, N : negative control and L : 100 bp ladder

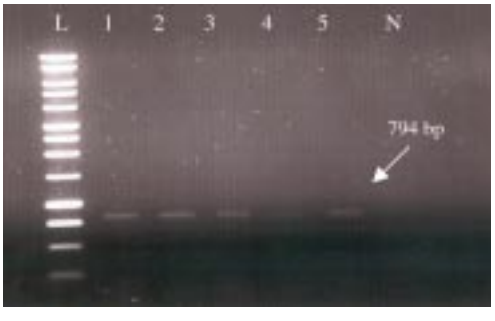

Plate 4. RT-PCR expression of PumPDS in the peel at 1, 2, 3, 4 and 5 months of fruit development, N : negative control & L : 1 Kb ladder

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CxparaPSY   MSVTLLWWS PNSQLSNCFG FVDSVREENR LFYSSRFLYQ HQTRTAVFNS  50
CunPSY      MSVTLLWWS PNSQLSNCFG FVDSVREENR LFYSSRFLYQ HQTRTAVFNS  50
CsinPSY     ---------- ---------- ---------- ---------- ----------   1
PumPSY      ---------- ---------- ---------- ---------- ----------   1

CxparaPSY   RPKQFNNSNK QRRNSYPLDT DLRHPCSSGI DLPEISCMVA STAGEVAMSS 100
CunPSY      RPKQFNNSNK QRRNSYPLDT DLRHPCSSGI DLPEISCMVA STAGEVAMSS 100
CsinPSY     ---------- ---------- DLRHPCSSGI DLPEISCMVA STAGEVAMSS  30
PumPSY      ---------- ---------- ---------- ---------- ----------   1

CxparaPSY   EEMVYNVVLK QAALVNKQPS GVTRDLDVNP DIALPGTLSL LSEAYDRCGE 150
CunPSY      EEMVYNVVLK QAALVNKQPS GVTRDLDVNP DIALPGTLSL LSEAYDRCGE 150
CsinPSY     EEMVYNVVLK QAALVNKQPS GVTRDLDVNP DIALPGTLSL LSEAYDRCGE  80
PumPSY      ---------- ---------- ---------- ---------- ----------   1

CxparaPSY   VCAEYAKTFY LGTLLMTSER RRAIWAIYVW CRRTDELVDG PNASHITPTA 200
CunPSY      VCAEYAKTFY LGTLLMTSER RRAIWAIYVW CRRTDELVDG PNASHITPTA 200
CsinPSY     VCAEYAKTFY LGTLLMTSER RRAIWAIYVW CRRTDELVDG PNASHITPTA 130
PumPSY      ---------- ---------- ---------- ---------- ----------   1

CxparaPSY   LDRWESRLED LFRGQPFDML DAALSDTVTK FPVDIQPFRD MIEGMRMDLR 250
CunPSY      LDRWESRLED LFRGRPFDML DAALSDTVTK FPVDIQPFRD MIEGMRMDLR 250
CsinPSY     LDRWESRLED LFRGRPFDML DAALSDTVTK FPVDIQPFRD MIEGMRMDLR 180
PumPSY      ---------- ---------- ---------- ---------- --- GMRMDFR   7

CxparaPSY   KSRYKNFDEL YLYCYYVAGT VGLMSVPVMG IAPDSQATTE SVYNAALALG 300
CunPSY      KSRYKNFDEL YLYCYYVAGT VGLMSVPVMG IAPDSQATTE SVYNAALALG 300
CsinPSY     KSRYKNFDEL YLYCYYVAGT VGLMSVPVMG IAPDSQATTE SVYNAALALG 230
PumPSY      KSRYKPFDEL YLYCYYVAGT VGLMSVPVMG IAPDSQATTE SVYNAALALG  57

CxparaPSY   IANQLTNILR DVGEDAPRGR VYLPQDELAQ AGLSDDDIFA GEVTIKWRNF 350
CunPSY      IANQLTNILR DVGEDARRGR VYLPQDELAQ AGLSDDDIFA GEVTIKWRNF 350
CsinPSY     IANQLTNILR DVGEDARRGR VYLPQDELAQ AGLSDDDIFA GEVTIKWRNF 280
PumPSY      IANQLTNILR DVGEDAQRGR VYLPQDELAQ AGLSDDDIFA GEVTNKWRNF 107

CxparaPSY   MKNQIKRARM FFDMAENGVT ELSEASRWPV WASLLLYRQI LDEIEANDYN 400
CunPSY      MKNQIKRARM FFDMAENGVT ELSEASRWPV WASLLLYRQI LDEIEANDYN 400
CsinPSY     MKNQIKRARM FFDMAENGV- ---------- ---------- ---------- 299
PumPSY      MKNQIKRARM FFDMAENGVT ELSEASRWPV WASLLLYRQI LDEIEA----- 153

CxparaPSY   NFTKRACVSK AKKIAALPIA YAKSLLRPSR IYTSKA436
CunPSY      NFTKRAYVSK AKKIAALPIA YAKSLLRPSR IYTSKA436
CsinPSY     ---------- ---------- ---------- ------- 299
PumPSY      ---------- ---------- ---------- ------- 153
```

*Figure 1. Comparison of the deduced partial amino acid sequence of **C. grandis** PSY (PumPSY) with **Citrus** x **Paradisi** PSY (CxParaPSY), **C. unshiu** PSY (CunPSY) and **C. sinensis** PSY (CsinPSY). The consensus sequence (shaded in black) was determined using the BioEdit Sequence Alignment Editor version 5.0.9*

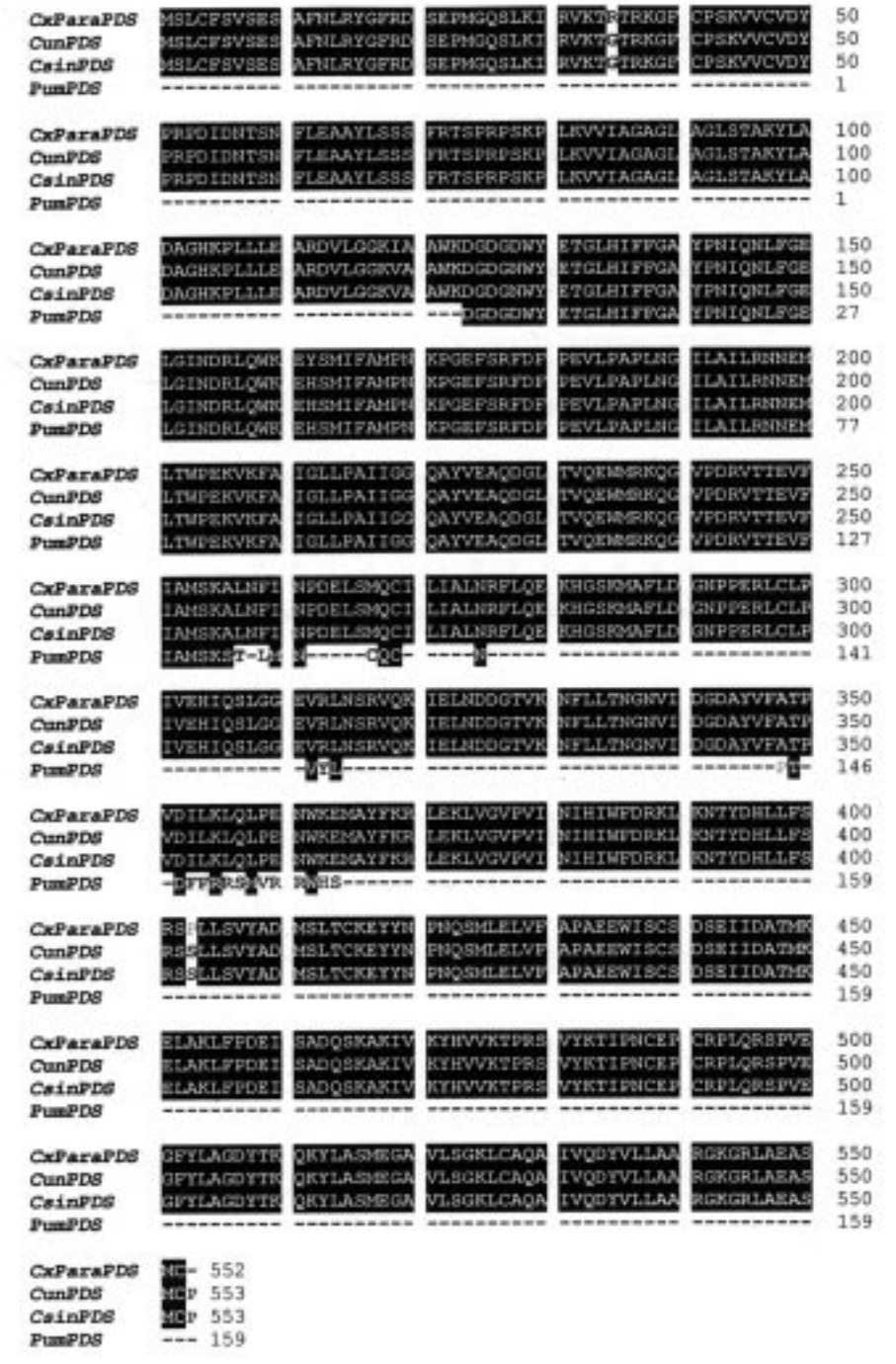

Figure 2. Comparison of the deduced partial amino acid sequence of ***C. grandis*** *PDS (PumPDS) with* ***Citrus*** *x* ***Paradisi*** *PDS (CxParaPDS),* ***C. unshiu*** *PDS (CunPDS) and* ***C. sinensis*** *PDS (CsinPDS). The consensus sequence (shaded black) was determined using the BioEdit Sequence Alignment Editor version 5.0.9*

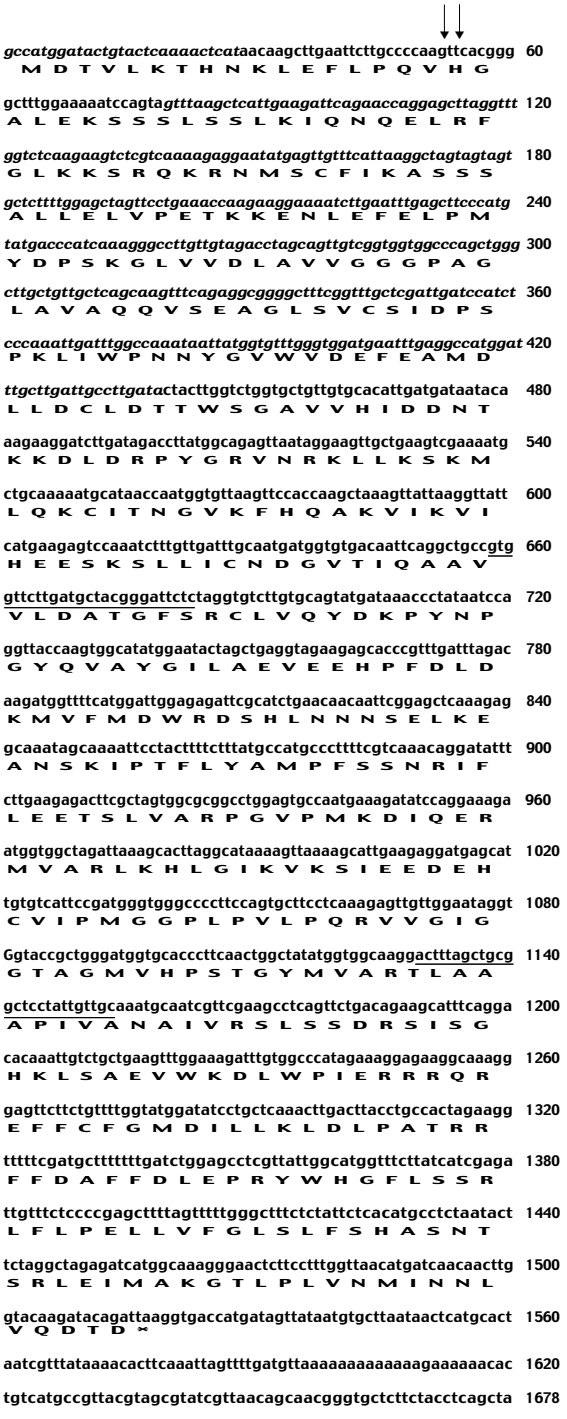

Figure 3. Nucleotide and amino acid sequence of ***C. grandis lycopene*** *β-cyclase (PumLYCb). Deduced full amino acid sequence is shown as single letters below the nucleotide sequence. The oligonucleotide primers used for the isolation of a partial clone are underlined. Putative cleavage sites are located at around arrows. A potential signal peptide (position 1-27) and NAD-binding site domain (position 80-442) are shown in italics*

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MDTVLKTHNKLEFLPQVHGALEKSSSLSSLKIQNQELRFGLKKSRQKRNMSCFIKASSSA
---HHH-----H--HHHH---HH------E-HHHHHHHHHH---------HEEEHH--HH

LLELVPETKKENLEFELPMYDPSKGLVVDLAVVGGGPAGLAVAQQVSEAGLSVCSIDPSP
HHEH---------HH-----------EEEEEE-------HHHHHHHHHH--EE-------

KLIWPNNYGVWVDEFEAMDLLDCLDTTWSGAVVHIDDNTKKDLDRPYGRVNRKLLKSKML
--E-------EE-HHHHHHHHH--------EEEE--------------HHHHHHHH--HH

QKCITNGVKFHQAKVIKVIHEESKSLLICNDGVTIQAAVVLDATGFSRCLVQYDKPYNPG
HEHH----HHHHHHHEEHHHHH---EEEE-----EEHEEEEH-----EHEE---------

YQVAYGILAEVEEHPFDLDKMVFMDWRDSHLNNNSELKEANSKIPTFLYAMPFSSNRIFL
-EEEE--HHHHH-------HHHHH------------HHH-------EEE--------EEE

EETSLVARPGVPMKDIQERMVARLKHLGIKVKSIEEDEHCVIPMGGPLPVLPQRVVGIGG
HHH-H----------HHHHHHHHHHHH--EE----H----E-----------EEEE---

TAGMVHPSTGYMVARTLAAAPIVANAIVRSLSSDRSISGHKLSAEVWKDLWPIERRRQRE
---EE------EEHHHH---HHHHHHEEHE------E------HHHH-----HH------

FFCFGMDILLKLDLPATRRFFDAFFDLEPRYWHGFLSSRLFLPELLVFGLSLFSHASNTS
HHH--HHHEHHH---HHHHHHH--H----HHH----------HHHHHHHHHHEE------

RLEIMAKGTLPLVNMINNLVQDTD
HHEEE------HHHHHH-------
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Figure 4. Secondary structure prediction of the deduced amino acid sequence of ***C. grandis*** *lycopene β-cyclase (PumLYCb). (H = helix, E = strand, - = no prediction)*

present in lower levels in the peel at the early developmental stages and later began to accumulate towards fruit maturation *(Plate 3)*. On the other hand, the intensity of the *PumPDS* mRNA signal was extremely low in the peel throughout fruit maturation compared with that of *PumPSY* and the *PumLYCb* transcript levels *(Plate 4)*. This low copy number of the *PDS* gene has also been reported in the Satsuma mandarin (Kita et al. 2001), green pepper fruit (Hugueney et al. 1992) and soybean (Bartley et al. 1991) and thus, may be a general biochemical feature of *PDS* genes in plants. In tomato, the *PDS* is known to be a single-copy gene (Giuliano et al. 1993; Corona et al. 1996).

The relative RTq-PCR results showed the expression profiles of *PumPSY* and *PumLYCb* to be similar to that obtained by RT-PCR results. The *PumPSY* product was first detected in the 1-month peel after 22 cycles, followed by the 5, 4, 2 and 3-month peel, respectively *(Figure 6)*. However, the *PumLYCb* product was first detected in the peel only after 27 cycles and at about the same time in all the stages of fruit development except for the second month which showed a lower expression *(Figure 7)*.

As fruit maturation progressed, the expression patterns of all three early carotenoid biosynthesis genes were different in the pummelo peel, indicating non-coordinate regulation. Such non-coordinate regulation has also been observed in Satsuma mandarin where the *CitPDS1* expression did not coincide with carotenoid accumulation in the peel. However, the induction of the *CitPSY1* gene toward maturation caused the peel colour of this citrus to change from green to orange, suggesting an important role of the *PSY* gene on the onset of carotenoid accumulation in citrus. Although there was a

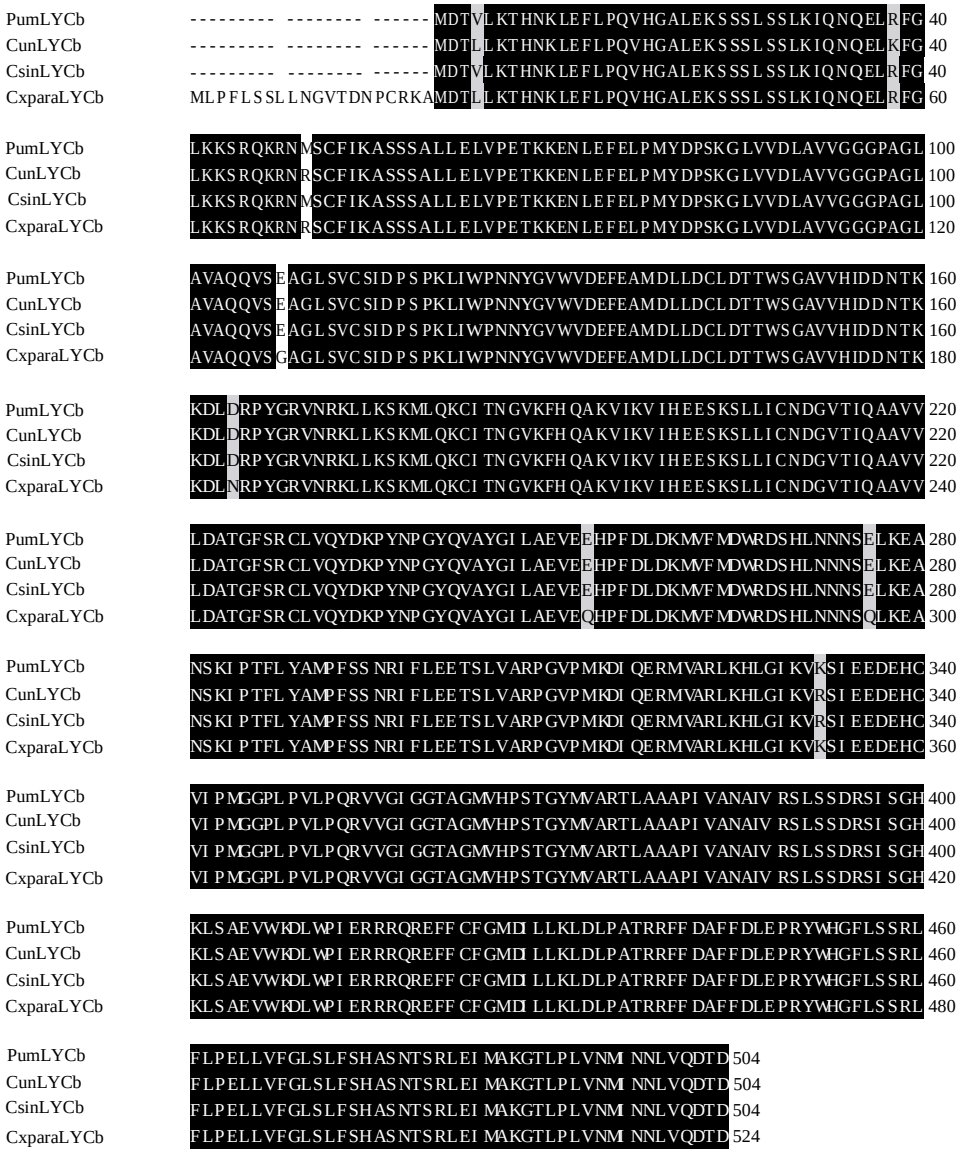

*Figure 5. Comparison of the deduced full amino acid sequence of **C. grandis** LYCb (PumLYCb) with **C. unshiu** LYCb (CunLYCb), **C. sinensis** LYCb (CsinLYCb) and **Citrus** x **Paradisi** LYCb (CxparaLYCb). The consensus sequence (shaded black) was determined using the BioEdit Sequence Alignment Editor version 5.0.9*

slight increase in the *PumPSY* and *PumLYCb* transcripts toward maturation, the expression patterns were not in accordance with carotenoid accumulation and chlorophyll disappearance leading to change in colouration of the peel from green to orange as observed in the Satsuma mandarin (*C. unshiu* Marc.) (Ikoma et al. 2001).

The presence of both the *lycopene β-* and *ε-cyclases* in the pummelo peel could also contribute to the lack of colouration from green to orange as these enzymes will convert lycopene to δ-carotenes instead of γ-carotenes which in turn will become converted to α-carotenes instead of β-carotenes. The α-carotenes will in turn be

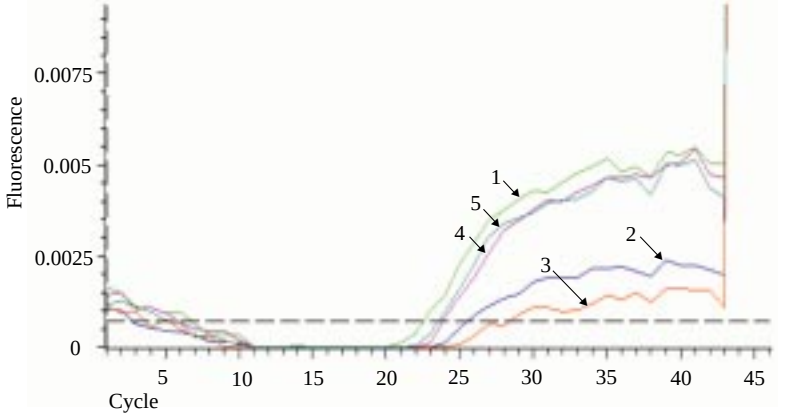

Figure 6. RT-qPCR profile of PumPSY expression in the peel at 1, 2, 3, 4 and 5 months of fruit development

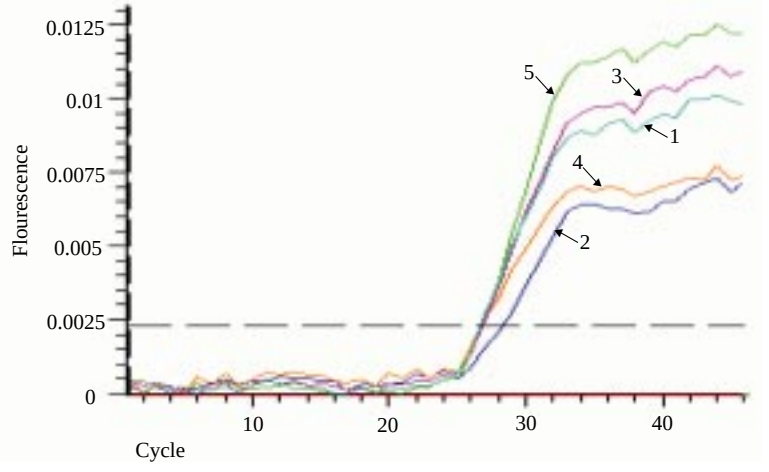

Figure 7. RTq-PCR profile of PumLYCb expression in the peel at 1, 2, 3, 4 and 5 months of fruit development

hydoxylated to lutein, resulting in the yellow pigment of the peel. Interestingly in tomato, it was found that the mRNA levels of *lycopene* β- and ε-*cyclases* which convert lycopene to γ or δ-carotenes respectively, during the 'breaker' stage, decline and completely disappear, apparently due to a down-regulation of these genes (Pecker et al. 1996; Ronen et al. 1999). Hence, the accumulation of lycopene (red pigment) in tomato fruits.

In addition, it was also observed that as fruit maturation progressed in the Satsuma mandarin and Valencia oranges, there was a simultaneous increase in the expression of other downstream carotenoid biosynthesis genes which led to a massive beta, beta-carotenoids accumulation like beta-cryptoxanthin and violaxanthin (Kato et al. 2004) that lead to orange pigmentation in the peel. Thus, it is essential to characterize the downstream synthesis genes of the carotenoid biosynthesis pathway as well, to better understand the expression of the carotenogenic genes in the pummelo peel during fruit maturation.

Conclusion

The results obtained in this study indicated that there is a distinctive expression profile

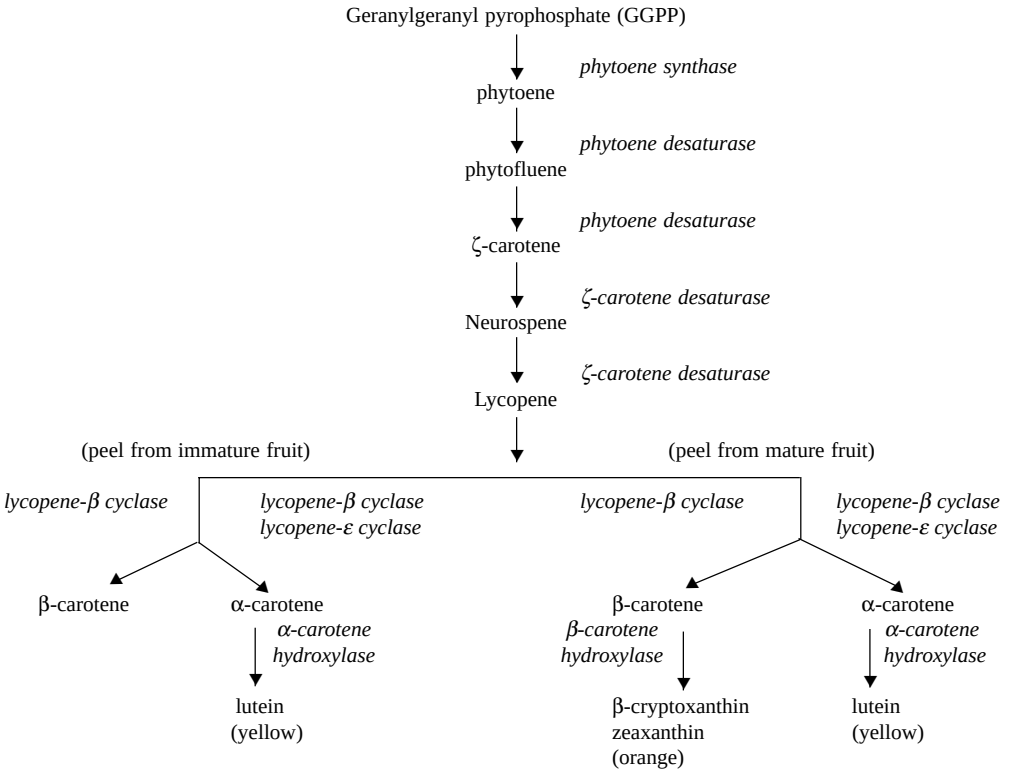

*Figure 8. Suggested pathway for carotenoid biosynthesis in the peel of immature (left) and mature (right) Satsuma mandarin (**Citrus unshiu** Marc.) fruit (Ikoma et al. 2001)*

of the early carotenoid genes in the pummelo peel compared to other plant species reported. The findings also suggest that the carotenoid biosynthesis in the Malaysian pummelo peel may follow an alternative branching pathway in comparison to that suggested for the Satsuma mandarin (*Citrus unshiu* Marc.) fruit (Ikoma et al. 2001) *(Figure 8).* These observations indicate that the primary mechanism controlling peel colour formation during citrus fruit development is based on the differential regulation of expression of carotenoid biosynthesis genes.

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References

Bartley, G.E., Viitanen, P.V., Pecker, I., Chamovitz, D., Hirschberg, J. and Scolnik, P.A. (1991). Molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway. *Proc. Natl. Acad. Sci. USA 88*: 6532–6

Bartley, G.E. and Scolnik, P.A. (1995). Plant carotenoids: Pigments for photoprotection, visual attraction and human health. *Plant Cell 7:* 1027–38

Bramley, P.M. (1985). The *in vitro* biosynthesis of carotenoids. *Adv. Lipid Res. 21:* 243–79

Corona, V., Aracri, B., Kosturkova, G., Bartley, G.E., Pitto, L., Giorgetti, L., Scolnik, P.A and

Giuliano, G. (1996) Regulation of a carotenoid biosynthesis gene promoter during plant development. *Plant J. 9:* 505–12

Cunningham, F.X. Jr. and Gantt, E. (1998). Genes and enzymes of carotenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Mol. Biol. 49:* 557–83

Cunningham, F.X. Jr., Pogson, B., Sun, Z., McDonald, K.A., DellaPenna, D. and Gant, E. (1996). Functional analysis of the β and ε-*lycopene cyclase* enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell 8:* 1613–26

Daito, H., Sato, Y., Hirose, K. and Umeda, K. (1975). Studies on maturity of citrus fruit. II. Changes in the colour and carotenoid group of pigments of Satsuma mandarin fruit during maturation. *Bull. Fruit Tree Res. Stn. B2:* 53–74

Giuliano, G., Bartley, G.E. and Scolnik, P.A. (1993). Regulation of carotenoid biosynthesis during tomato development. *Plant Cell 5:* 379–87

Hugueney, P., Romer, S., Kuntz, M. and Camara, B. (1992). Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and z-carotene in *Capsicum* chromoplasts. *Eur. J. Biochem. 209:* 399–407

Ikoma, Y., Komatsu, A., Kita, M., Ogawa, K., Omura, M., Yano, M. and Moriguchi T. (2001). Expression of a *phytoene synthase* gene and characteristic carotenoid accumulation during citrus fruit development. *Physiol. Plant 111:* 232–8

Kashim, M.S., Vellupillai, M., Hassan, A. and Hashim, M. (2005). Detection and analysis of carotenoid pigments in the Malaysian pummelo *(Citrus grandis)* fruit peel using reverse-phase high performance liquid chromatography (RP-HPLC). (Submitted to J. Trop. Agric. And Fd. Sc.)

Kato, M., Ikoma, Y., Matsumoto, H., Sugiura, M., Hyodo, H. and Yano, M. (2004). Accumulation of carotenoids and expression of carotenoid biosynthesis during maturation in citrus fruit. *Plant Physiol. 134(2):* 824–37

Kita, M., Komatsu, A., Omura, M., Yano, M., Ikoma, Y. and Moriguchi, T. (2001) Cloning and expression of CitPDS1, a gene encoding *phytoene desaturase* in citrus. *Biosci. Biotechnol. Biochem. 65:* 1424–8

Li, Z-H., Matthews, P.D., Burr, B. and Wurtzel, E.T. (1996) Cloning and characterization of a maize cDNA encoding *phytoene desaturase*, an enzyme of the carotenoid biosynthetic pathway. *Plant Mol. Biol. 30:* 269–79

Matsumura, H., Nirasawa, S. and Terauchi, R. (1999). Transcript profiling in rice (*Oryza sativa*) seedlings using serial analysis of gene expression (SAGE). *Plant J. 20(6):* 719–26

Olson, J.A. (1989). Provitamin-A function of carotenoids: The conversion of β-carotene into vitamin-A. *J. Nutr. 119:* 105–8

Pecker, I., Gabbay, R., Cunningham, FX Jr. and Hirschberg, J. (1996). Cloning and characterization of the cDNA for *lycopene beta-cyclase* from tomato reveals a decrease in its expression during fruit ripening. *Plant Mol. Biol. 30(4):* 807–19

Ronen, G., Cohen, M. and Zamir, D. (1999). Regulation of carotenoid biosynthesis during tomato fruit development: Expression of the gene for *lycopene epsilon-cyclase* is down-regulated during ripening and is elevated in the mutant. *Delta Plant J. 17:* 341–51

Stewart, I. (1977). Provitamin A and carotenoid content of citrus juices. *J. Agric. Food Chem. 25:* 1132–7

Tsushima, M., Maoka, T., Katsuyama, M., Kozuka, M., Matsuno, T., Tokuda, H., Nishino, H., Iwashima, A. (1995). Inhibitory effect of natural carotenoids on Epstein-Barr virus activation activity of a tumor promoter in Raji cells. A screening study for anti-tumor promoters. *Biol. Pharm. Bull. 18:* 227–33

von Lintig, J., Welsch, R., Bonk, M., Guiliano, G., Batschauer, A., Kleinig, H. (1997). Light-dependent regulation of carotenoid biosynthesis occurs at the level of *phytoene synthase* expression and is mediated by phytochrome in *Sinapis alba* and *Arabidopsis thaliana* seedlings. *Plant J. 12:* 625–34

Abstrak

Tiga klon separa cDNA berbeza yang mengekodkan enzim *pummelo phytoene synthase (PumPSY), pummelo phytoene desaturase (PumPDS)* dan *pummelo lycopene-beta-cyclase (PumLYCb)* di peringkat awal tapak jalan biosintesis karotenoid telah dipencilkan daripada kulit buah limau bali (*Citrus grandis* cv. Melomas). Perbandingan jujukan asid amino ketiga-tiga gen dari kulit limau bali ini menunjukkan lebih daripada 90% persamaan dengan Satsuma mandarin (*Citrus unshiu* Marc.), *Citrus* x *Paradisi* and *Citrus sinensis.*

Kajian ekspresi gen pula menunjukkan transkrip *PumPSY* adalah yang tertinggi di dalam kulit pada awal tumbesaran buah, menurun pada bulan ketiga dan kemudian meningkat semula semasa kematangan buah. Manakala transkrip *PumLYCb* didapati rendah pada awal tumbesaran buah dan kemudian meningkat semula semasa kematangan. Transkrip *PumPDS* berada dalam kuantiti yang sangat rendah pada sepanjang tumbesaran buah berbanding dengan *PumPSY* dan *PumLYCb*. Kajian ekspresi gen ini menunjukkan tiada saling kaitan antaranya dan tidak mengikuti corak seperti yang terdapat di dalam buah citrus lain, yang menunjukkan penambahan karotenoid dan kekurangan klorofil diikuti dengan perubahan warna kulit daripada hijau kepada oren seperti kulit buah Satsuma mandarin dan Valencia.

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