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 μ g 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, USA) 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 quantitativepolymerase 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

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	LGTLLMIS ER	RRAIWAIYVW	CRRTDELV DG	P NAS HI TP TA	200
CunPSY	VCAEYAKTFY	LGTLLMTS ER	RRAIWAIYVW	CRRTDELVDG	P NAS HI TP TA	200
CsinPSY	VCAEYAKTFY	LGTLLMTS ER	RRAIWAIYVW	CRRTDELV DG	P NAS HI TP TA	130
PumPSY						1
CxparaPSY	L DRWES R LED	LFRGQPFDML	DAALS DTVTK	FPVDIQPFRD	MIEGMRMDLR	250
CunPSY	L DRWES RLED	LFRGRPFDML	DAALS DTVTK	FPVDIQPFRD	MIEGMRMDLR	250
CsinPSY	L DRWES RLED	LFRGRPFDML	DAALS DTVTK	FPVDIQPFRD	MIEGMRMDLR	180
PumPSY					GMRMD <mark>F</mark> R	7
CxparaPSY	KS R YKNFD EL	YLYCY YVAGT	VGLMSVPVMG	IAPDSQATTE	SV YNAALALG	300
CunPSY	KSRYKNFDEL	YLYCY YVAGT	VGLMSVPVMG	IAPDSQATTE	SVYNAALALG	300
CsinPSY	KSRYKNFDEL	YLYCY YVAGT	VGLMSVPVMG	IAPDSQATTE	SVYNAALALG	230
PumPSY	KS R YK <mark>P</mark> F DEL	YLYCY YVAGT	VGLMSVPVMG	IAPDSQATTE	SVYNAALALG	57
CxparaPSY	IANQLT NI LR	DVGEDA <mark>P</mark> RGR	VYLPQDELAQ	AGLSDDDIFA	GEVTIKWRNF	350
CunPSY	IANQLTNILR	DVGEDARRGR	VYLPQDELAQ	AGLSDDDIFA	GEVTIKWRNF	350
CsinPSY	IANQLTNILR	DVGEDARRGR	VYLPQDELAQ	AGLSDDDIFA	GEVTIKWRNF	280
PumPSY	IANQLTNILR	DVGEDAQRGR	VYLPQDELAQ	AGLSDDDIFA	GEVT <mark>N</mark> KWRNF	107
CxparaPSY	MKNQIKRARM	FFDMAENGVT	ELSEASRWPV	WASLLLYRQI	L DE I E ANDYN	400
CunPSY	MKNQIKRARM	F F DMAE NG VT	ELSEASRWPV	WASLLLYRQI	L DE I E ANDYN	400
CsinPSY	MKNQIKRARM	FFDMAENGV-				299
PumPSY	MKNQIKRARM	FDMAENGVT	ELSEASRWPV	WASLLLYRQI	LDEI EA	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

Expression of peel carotenoid genes during pummelo fruit maturation

CxParaPDS CunPDS CsinPDS FumPDS	MSLCPSVSES MSLCPSVSES MSLCPSVSES	AFNLRYGFRD AFNLRYGFRD AFNLRYGFRD	SEPHGQSLKI SEPHGQSLKI SEPHGQSLKI	RVKTRTRKOF RVKTOTRKOF RVKTOTRKOF	CPSKVVCVDY CPSKVVCVDY CPSKVVCVDY	50 50 50 1
CxParaPDS CunPDS CsinPDS PumPDS	PRPOIDNTSN PRPOIDNTSN PRPOIDNTSN	FLEAAYLSSS FLEAAYLSSS FLEAAYLSSS	FRTSPRPSKP FRTSPRPSKP FRTSPRPSKP	LRVVIAGAGL LRVVIAGAGL LRVVIAGAGL	aglstaryla Aglstaryla Aglstaryla	100 100 100 1
CxParaPDS CunPDS CainPDS PumPDS	DAGHRPLLLE DAGHRPLLLE DAGHRPLLLE	ARDVLOGKIN ARDVLGGRVA ARDVLGGRVA	NNKDGDGDWY ANKDGDGNWY ANKDGDGNWY DGDGDWY	ETGLHIFFGA ETGLHIFFGA ETGLHIFFGA ETGLHIFFGA	Abhiðnrach Abhiðnrach Abhiðnrach Abhiðnrach	150 150 150 27
CxParaPDS CunPDS CsinPDS PumPDS	lgindrlqwk Lgindrlqwk Lgindrlqwk Lgindrlqwk	EYSMIFAMPN EHSMIFAMPN EHSMIFAMPN EHSMIFAMPN	KPGEPSRFDP KPGEPSRFDP KPGEPSRFDP KPGEPSRFDP	PEVLPAPLNG PEVLPAPLNG PEVLPAPLNG PEVLPAPLNG	ILAILRNNEH ILAILRNNEH ILAILRNNEH ILAILRNNEH	200 200 200 77
CxParaPDS CunPDS CsinPDS FumPDS	LTMPERVRFA LTMPERVRFA LTMPERVRFA LTMPERVRFA	IGLLPAIIGG IGLLPAIIGG IGLLPAIIGG IGLLPAIIGG	QAYVEAQDGL QAYVEAQDGL QAYVEAQDGL	tvoenerkog tvoenerkog tvoenerkog	VPDRVTTEVF VPDRVTTEVF VPDRVTTEVF VPDRVTTEVF	250 250 250 127
CxParaPDS CunPDS CsinPDS FumPDS	IANSKALNFI IANSKALNFI IANSKALNFI IANSKS <mark>T-L</mark> H	NFDELSMQCI NFDELSMQCI NFDELSMQCI NCQC	LIALNRFLQE LIALNRFLQE LIALNRFLQE N	KHGSIMAFLD KHGSIMAFLD KHGSIMAFLD	GNPPERLCLP GNPPERLCLP GNPPERLCLP	300 300 300 141
CxParaPDS CunPDS CsinPDS FumPDS	IVENIQSLGG IVENIQSLGG IVENIQSLGG	evrlnsrvok evrlnsrvok evrlnsrvok MBL	IELNDOGTVK IELNDOGTVK IELNDOGTVK	NFLLTNGNVI NFLLTNGNVI NFLLTNGNVI	DGDAYVFATF DGDAYVFATF DGDAYVFATF	350 350 350 146
CxParaPDS CunPDS CsinPDS PumPDS	VDILKLQLPE VDILKLQLPE VDILKLQLPE -DYFRESVR	nwkenayfkr Inwkenayfkr Inwkenayfkr Inwkenayfkr	LEKLVGVPVI LEKLVGVPVI LEKLVGVPVI	NIHIWPDRKL NIHIWPDRKL NIHIWPDRKL	KNTYDHLLFS KNTYDHLLFS KNTYDHLLFS	400 400 400 159
CxParaPDS CunPDS CsinPDS PumPDS	RSPLLSVYAD RSSLLSVYAD RS <mark>S</mark> LLSVYAD	MSLTCREYYN MSLTCREYYN MSLTCREYYN	PNQSMLELVP PNQSMLELVP PNQSMLELVP	APAEEWISCS APAEEWISCS APAEEWISCS	DSEIIDATME DSEIIDATME DSEIIDATME	450 450 450 159
CxParaPDS CunPDS CsinPDS FumPDS	ELAKLFFDEI ELAKLFFDEI ELAKLFFDEI	SADQSKAKIV SADQSKAKIV BADQSKAKIV	KYHVVKTPRS KYHVVKTPRS KYHVVKTPRS	VYRTIPNCEP VYRTIPNCEP VYRTIPNCEP	CRPLQRSPVE CRPLQRSPVE CRPLQRSPVE	500 500 500 159
CxParaPDS CunPDS CsinPDS PumPDS	GFYLAGDYTR GFYLAGDYTR GFYLAGDYTR	QKYLASMEGA QKYLASMEGA QKYLASMEGA	VLSGKLCAQA VLSGKLCAQA VLSGKLCAQA	IVQDYVLLAA IVQDYVLLAA IVQDYVLLAA	RGKGRLAEAS RGKGRLAEAS RGKGRLAEAS	550 550 550 159
CxParaPDS CunPDS CsinPDS PumPDS	40 - 552 40 p 553 40 p 553 159					

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

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gecatggatactgtactcaaaactcataacaagcttgaattcttgccccaagttcacggg MDTVLKTHNKLEFLPQVHG	60
gctttggàààààtccàgtàgtttaagctcattgaagattcagaaccaggagcttaggttt ALEKSSSLSSLKIQNQELRF	120
ggteteaagaagtetegteaaaagaggaatatgagttgttteattaaggetagtagtag GLKKSRQKRNMSCFIKASSS	180
gctcttttggagctagtteetgaaaceaagaaggaaaatettgaatttgagetteeeatg ALLELVPETKKENLEFELPM	240
$ \begin{array}{cccc} tatgacccatcaaagggccttgttgtagacctagcagttgtcggtggtggcccagctggg\\ \mathbf{Y} \ \mathbf{D} \ \mathbf{P} \ \mathbf{S} \ \mathbf{K} \ \mathbf{G} \ \mathbf{L} \ \mathbf{V} \ \mathbf{V} \ \mathbf{D} \ \mathbf{L} \ \mathbf{A} \ \mathbf{V} \ \mathbf{V} \ \mathbf{G} \ \mathbf{G} \ \mathbf{G} \ \mathbf{P} \ \mathbf{A} \ \mathbf{G} \end{array} $	300
cttgctgttgctcagcaagtttcagagggggggtttcggtttgctcgattgatccatct L A V A Q Q V S E A G L S V C S I D P S	360
сссаааttgatttggccaaataattatggtgtttgggtggatgaatttgaggccatggat РКLІWРNNYGVWVDEFEAMD	420
<i>ttgettgattgeettgata</i> ctacttggtctggtgctgttgtgcacattgatgataataca L L D C L D T T W S G A V V H I D D N T	480
аадааддаtcttgatagaccttatggcagagttaataggaagttgctgaagtcgaaaatg ККОLОКРҮСКVNККLLКЅКМ	540
ctgcaaaaatgcataaccaatggtgttaagttcaccaagctaaagttattaaggttatt L Q К С I Т N G V К F H Q A К V I К V I	600
catgaagagtccaaatctttgttgatttgcaatgatggtgtgacaattcaggctgcc <u>gtg</u> НЕЕЅКЅLLICNDGVTIQAAV	660
gttcttgatgctacgggattctctaggtgtcttgtgcagtatgataaaccctataatcca VLDATGFSRCLVQYDKPYNP	720
ggttaccaagtggcatatggaatactagctgaggtagaagagcacccgtttgattagac GYQVAYGILAEVEEHPFDLD	780
aagatggttttcatggattggagagattcgcatctgaacaacaattcggagctcaaagag KMVFMDWRDSHLNNNSELKE	840
gcaaatagcaaaattcctacttttctttatgccatgcccttttcgtcaaacaggatattt ANSКIPTFLYAMPFSSNRIF	900
cttgaagagacttcgctagtggcgcggcctggagtgccaatgaaagatatccaggaaaga LЕЕТЅLVАКРGVРМКDIQЕК	960
atggtggctagattaaagcacttaggcataaaagttaaaagcattgaagaggatgagcat M V A R L K H L G I K V K S I E E D E H	1020
tgtgtcattccgatgggtggggccccttccagtgcttcctcaaagagttgttggaataggt СVІРМGGРLРVLРQRVVGІG	1080
Ggtaccgctgggatggtgcacccttcaactggctatatggtggcaagg <u>actttagctgcg</u> G T A G M V H P S T G Y M V A R T L A A	1140
gctcctattgttgcaaatgcaatcgttcgaagcctcagttctgacagaagcatttcagga APIVANAIVRSLSSDRSISG	1200
cacaaattgtctgctgaagtttgggaaagatttgtggcccatagaaaggaagg	1260
gagttcttctgttttggtatggatatcctgctcaaacttgacttacctgccactagaagg ЕГГСГСМ DІLLКLDLРАТКК	1 320
tttttcgatgctttttttgatctggagcctcgttattggcatggtttcttatcatcgaga FFDAFFDLEPRYWHGFLSSR	1 380
ttgtttctccccgagcttttagtttttgggctttctctattctcacatgcctctaatact LFLPELLVFGLSLFSHASNT	1440
tctaggctagagatcatggcaaagggaactcttcctttggttaacatgatcaacaacttg SRLEIMAKGTLPLVNMINNL	1500
gtacaagatacagattaaggtgaccatgatagttataatgtgcttaataactcatgcact V Q D T D =	1560
aatcgtttataaaacacttcaaattagttttgatgttaaaaaaaa	1620
tgtcatgccgttacgtagcgtatcgttaacagcaacgggtgctcttctacctcagcta	1678

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

MDTVLKTHNKLEFLPQVHGALEKSSSLSSLKIQNQELRFGLKKSRQKRNMSCFIKASSSA HHHHHHHHHHE-HHHHHHHHHH
LLELVPETKKENLEFELPMYDPSKGLVVDLAVVGGGPAGLAVAQQVSEAGLSVCSIDPSP HHEHHHEEEEEEHHHHHHHHHH-EE
KLIWPNNYGVWVDEFEAMDLLDCLDTTWSGAVVHIDDNTKKDLDRPYGRVNRKLLKSKML EEE-HHHHHHHHHHH
QKCITNGVKFHQAKVIKVIHEESKSLLICNDGVTIQAAVVLDATGFSRCLVQYDKPYNPG HEHHHHHHHHHEEHHHHHEEEEEEHEEEEHEHEE
YQVAYGILAEVEEHPFDLDKMVFMDWRDSHLNNNSELKEANSKIPTFLYAMPFSSNRIFL -EEEEHHHHHHHHHHHHHEEEEEE
EETSLVARPGVPMKDIQERMVARLKHLGIKVKSIEEDEHCVIPMGGPLPVLPQRVVGIGG HHH-HEHHHHHHHHHHHHHH-EEHEEEEE
TAGMVHPSTGYMVARTLAAAPIVANAIVRSLSSDRSISGHKLSAEVWKDLWPIERRRQRE EEEEHHHHHHHHHHEEHEEHHHHHH
FFCFGMDILLKLDLPATRRFFDAFFDLEPRYWHGFLSSRLFLPELLVFGLSLFSHASNTS HHHHHHEHHHHHHHHHHHHHHHHHHHHH

RLEIMAKGTLPLVNMINNLVQDTD HHEEE-----HHHHHH------

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 noncoordinate 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

PumLYCb	MDTVLKTHNKLEFLPQVHGALEKSSSLSSLKIQNQELRFG 40
CunLYCb	
CsinLYCb	
CxparaLYCb	MLPFLSSLLNGVTDNPCRKA <mark>MDTLLKTHNKLEFLPQVHGALEKSSSLSSLKIQNQEL</mark> RFG 60
PumI YCh	I KKSROKRN <mark>y</mark> SCEIKASSSALLEI VPETKKENI EEEI PMYDPSKGI VVDI AVVGGGPAGI 100
CunLYCb	I KKSROKRNRSCEIKASSSALLELVPETKKENI EEELPMYDPSKGLVVDLAVVGGGPAGL 100
CsinLYCh	
CxparaLYCb	LKKSRQKRNISCH KASSSALLELVPETKKEN LEFELP MYDPSKG LVVDLAVVGGGPAGL 120 LKKSRQKRNRSCF I KASSSALLE LVPETKKEN LEFELP MYDPSKG LVVDLAVVGGGPAGL 120
PumI VCh	AVAOOVSEAGE SVC SID & S ØKT I WØNNYGVWVDEFF AMDT I DOL DT TWSGAVVHIDDN TK 160
CunI YCh	AVAQOVSEAGUSVCSIDISTREUVI ANTOVI V DELEAMDEEDCEDITIVSOAV HIDDATK 160
CsinI VCh	AVAQUE A GLEVCSIDIST KLIWI NNIGY WYDEFEAMDLLDCLDT I WSOAY HIDDN IK 160
Cynaral VCh	AVAQUYSEAGESVCSIDISIKEIWINNIGYWVDEFEAMDLEDCEDIIWSGAVVHIDDNITK 100
CAPARAL I CO	AVAQQV50A0ESVCSIDFSFKEIWFNNIOV WVDEFEAMDEEDCEDFFWSOAVVHIDDNTK 180
PumLYCb	KDL <mark>D</mark> RPYGRVNRKLLKSKMLQKCITNGVKFHQAKVIKVIHEESKSLLICNDGVTIQAAVV220
CunLYCb	KDL <mark>D</mark> RP YGR VNRKLLKS KMLQKCI TN GVKFH QA K V I KV I HEES KSLLI CNDG V T I QAA V V 220
CsinLYCb	KDL <mark>D</mark> RP YGR VNRKLLKS KMLQKCI TN GVKFH QA K V I KV I HEES KSLLI CNDG V T I QAA V V 220
CxparaLYCb	KDL <mark>N</mark> RPYGRVNRKLLKSKMLQKCI TNGVKFH QAKVIKV IHEESKSLLICNDGVT IQAAVV 240
PumLYCb	LDATGFSR CLVQYDKP YNP GYQVAYGI LAE VE <mark>E</mark> HP FDLDKM/FMDWRDSHLNNNS <mark>E</mark> LKEA 280
CunLYCb	LDATGFSR CLVQYDKP YNP GYQVAYGI LAEVEEHPF DLDKMVF MDWRDS HLNNNSELKEA 280
CsinLYCb	LDATGFSR CLVQYDKPYNPGYQVAYGI LAEVE <mark>E</mark> HPFDLDKMVFMDWRDSHLNNNS <mark>E</mark> LKEA 280
CxparaLYCb	LDATGFSR CLVQYDKPYNPGYQVAYGI LAEVE <mark>Q</mark> HPFDLDKMvFMDwRDSHLNNNS <mark>Q</mark> LKEA 300
PumLYCb	NSKI PTFL YAMPFSS NRI FLEETSL VARPGVPMKDI QERMVARLKHLGI KV <mark>K</mark> SI EEDEHC 340
CunLYCb	NSKI PTFL YAMPFSS NRI FLEETSLVARPGVPMKDI OERMVARLKHLGI KVRSI EEDEHC 340
CsinLYCb	NSKI PTFL YAMPFSS NRI FLEETSLVARPGVPMKDI OERMVARLKHLGI KVRSI EEDEHC 340
CxparaLYCb	NSKI PTFL YAMPFSS NRI FLEETSLVARPGVPMKDI QERMVARLKHLGI KV <mark>K</mark> SI EEDEHC 360
PumLYCb	VI PMGGPL PVLPORVVGI GGTAGM/HPSTGYM/ARTLAAAPI VANAIV RSLSSDRSI SGH 400
CunLYCb	VI PMGGPL PVLPORVVGI GGTAGMVHPSTGYMVARTLAAAPI VANAIV RSLSSDRSI SGH 400
CsinLYCb	VI PMGGPL PVLPORVVGI GGTAGMVHPSTGYMVARTLAAAPI VANAIV RSLSSDRSI SGH 400
CxparaLYCb	VI PMGGPL PVLPQRVVGI GGTAGM/HPSTGYM/ARTLAAAPI VANAIV RSLSSDRSI SGH 420
PumLYCb	KLSAEVWKDLWPIERRROREFFCFGMDLLLKLDLPATRRFFDAFFDLEPRYWHGFLSSRL460
CunLYCb	KLS AE VWKOLWPI ER RROREFF CF GMDILLKLDLP AT RRFF DAFF DLE PRYWHGFLS SRL 460
CsinLYCb	KLS AE VWKOLWPI ER RROREFF CF GMDILLKLDLP AT RRFF DAFF DLE PRYWHGFLS SRL 460
CxparaLYCb	KLS AE VWKOLWPI ERRRQREFF CFGMDILLKLDLPATRRFF DAFFDLE PRYWHGFLS SRL 480
PumLYCb	FLPELLVFGLSLFSHASNTSRLEI MAKGTLPLVNM NNLVODTD 504
CunLYCb	FLPELLVFGLSLFSHASNTSRLEI MAKGTLPLVNM NNLVODTD 504
CsinLYCb	FLPELLVFGLSLFSHASNTSRLEI MAKGTLPLVNM NNLVODTD 504
CxparaLYCb	FLPELLVFGLSLFSHASNTSRLEI MAKGTLPLVNM NNLVQDTD 524
-	

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 Expression of peel carotenoid genes during pummelo fruit maturation



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, betacarotenoids accumulation like betacryptoxanthin 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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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.