Expression Patterns of Genes Involved in Carotenoid Biosynthesis in Pepper

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To study the regulatory mechanism of isoprenoid (carotenoid) biosynthesis, we have compared the expression patterns of nine isoprenoid biosynthetic genes in Korean red pepper (*Capsicum. annuum* cv. NocKaung). The expression of geranylgeranyl pyrophosphate synthase gene was initially induced at early ripening stage (I1) and was rather slightly decreased during pepper fruit ripening. The ex-pression of phytoene synthase gene was strongly induced at semi-ripening stage (I2) and the phytoene desaturase transcript was maximally induced at the fully ripened stage (R). Our results suggest that genes encoding two 3-hydroxy-3-methylglutaryl-CoA reductase isozymes (HMGR1 and HMGR2) and farnesyl pyrophosphate synthase might be not so critical in pepper carotenoid biosynthesis but three genes encoding geranylgeranyl pyrophosphate synthase, phytoene synthase and phytoene desaturase were induced in a sequential manner and coordinately regulated during the ripening of pepper fruit.

Key words : *pepper* (Capsicum annuum), *isoprenoids, carotenoids.*

In plants, the isoprenoid pathway is responsible for the synthesis of phytosterols, phytoalexin, carotenoids, some phytohormones and other isoprene-containing compounds.[1,2] The activities of enzyme HMGR (EC1.1.1.34) catalyzing the conversion of HMG-CoA into mevalonate in the first committed step of isoprenoid pathway were detected in several compartments, which include the plastid for carotenoid synthesis, the endoplasmic reticulum cytosol for sterol and phytoalexin synthesis, and the mitochondria for the synthesis of the side chain of ubiquinone.[3,4] Camara *et al.* reported that a chloroplast-localized HMGR activity could be responsible for carotenoid synthesis in pepper.[5] But, Narita *et al.* noticed that the increase in carotenoid synthesis during tomato fruit ripening occurs without a corresponding increase in either cytosolic or plastidic HMGR activities and tomato HMGR is required only at early stage of fruit development.[6] Recently, HMGR independent plastidic GAP-pyruvate pathway was suggested instead of previously accepted HMGR dependent acetate-mevalonate pathway for the synthesis of IPP as a building block of carotenoid synthesis. It is, however, still too early to overestimate the importance of the discovery of the GAP-pyruvate pathway for isoprenoids biosynthesis in plant.[7] The importance of a regulated HMGR activity in controlling iso-prenoid metabolism including carotenoid biosynthesis is still controversial.

Carotenoids are a diverse group of pigments that are widely distributed in nature and found in all photosynthetic organisms.[8,9] Carotenoids synthesized and accumulated in plastids play a crucial role in light harvesting and are essential photoprotective agents in photosynthesis. They are often responsible for the red, orange and yellow colors of fruits and flowers. They are also precursors of the abscisic acid and important components of mammalian diet as a source of vitamin A.[10,11]

In this study, Korean red pepper was used as a model system to investigate the molecular regulation of nine genes encoding two HMGR isozymes and seven enzymes involved in isoprenoid biosynthetic pathway (Fig. 1).[3,12]

Materials and methods

Plant materials. Pepper (*Capsicum. annum* cv. NocKaung) seeds were purchased from JungAng Seed Company and grown under greenhouse conditions. For RNA isolation, two-month-old pepper plants were divided into four different parts such as leaf, stem, root and flower. Mature green fruits and red ripe fruits were harvested at 35 and 50 DAF, respectively. According to the degree of fruit ripening, pepper fruits of four stages, mature green, intermediate, intermediate, and red, were harvested between 30~50 DAF. All harvested

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Abbreviations: CCS, capsanthin/capsorubin synthase; DAF, days after flowering; FPS, farnesyl pyrophosphate synthase; GAP, glyceraldehyde-3-phosphate; GGPS, geranylgeranyl pyrophosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl pyrophosphate; LCY(b), lycopene-β-cyclase; LCY(e), lycopene-ε-cyclase; ORF, open reading frame; PCR, polymerase chain reaction; PDS, phytoene desaturase; PSY, phytoene synthase; RT, reverse transcriptase.

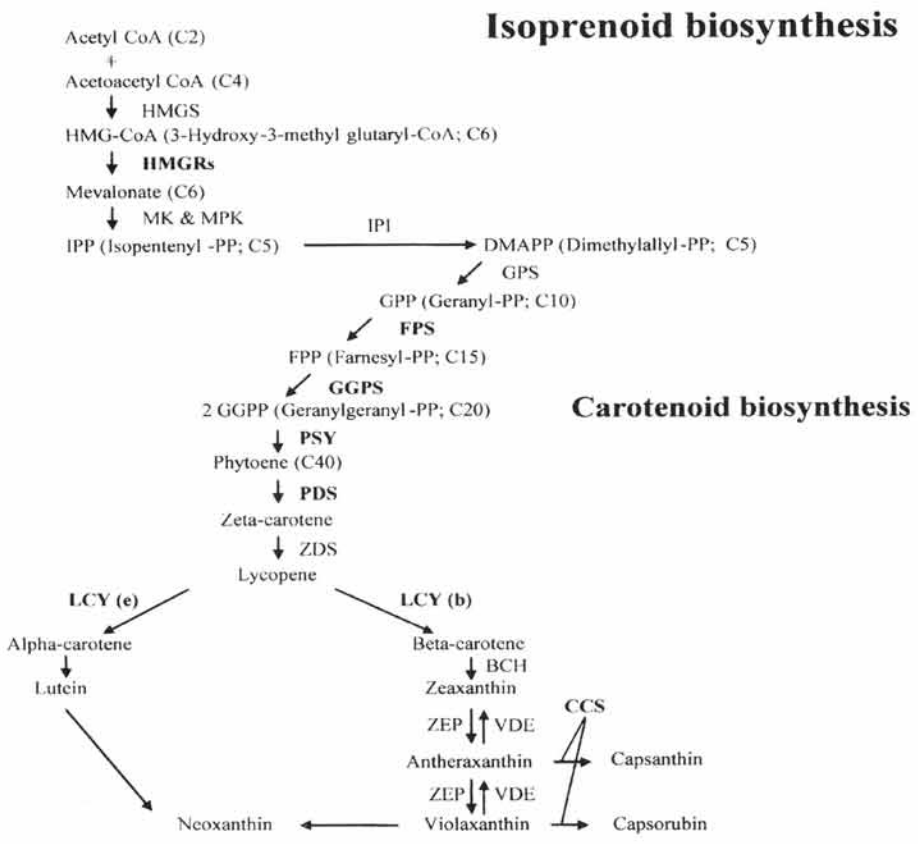

Fig. 1. Outline of carotenoid biosynthetic pathway in pepper. Enzymes whose cDNAs have been used in this study are bolded. Not all intermediate are shown. HMGS: 3-hydroxy-3-methylglutary-CoA synthase, MK & MPK: mevalonate kinate & mevalonate PP kinate, IPI: isopentenyl PP isomerase, GPS: geranyl PP synthase, ZDS: zeta-carotene desaturase, BCH: beta-carotene hydroxylase, ZEP: zeaxanthin epoxidase, VDE: violaxanthin deepoxidase.

samples were quickly frozen in a liquid N_2 and stored at -80°C for further analysis.

PCR for cloning of *Fps, Ggps, Psy, Pds, Lcy(b), Lcy(e),* and *Ccs* cDNAs. To obtain cDNA fragments of isoprenoids biosynthetic genes, several PCRs were carried out as summarized in Table 1. The simple PCRs for cloning of *Fps* and *Ggps* were performed for 30 cycles with each DNA template and 0.2 μM of a pair of primer per each gene. After preheating at 99°C (10 min) and chilling of DNA templates, each cycle was done at 94 for denaturation (30 sec), 60°C for annealing (1 min), and 72°C for extension (1.5 min) with *Ex-taq* polymerase (Takara). The RT-PCR technique was used for cloning of *Psy, Pds, Lcy(b), Lcy(e),* and *Ccs*. After synthetic step of cDNAs by RT reaction for 20 min at 55°C and denaturation step for 5 min at 99°C with RNA template and 1 μM of a reverse primer, further PCR was followed for 30 cycles with 1 μl of a reverse transcriptase reaction product containing cDNA fragments as a template and 1 μM of a forward primer. PCR cycles were done at 94°C for denaturation (30sec), 60°C for annealing (30 sec, 1 min or 1.5 min), and 72°C for extension (1.5 min) with RNA PCR kit (Takara). All PCR products were subcloned into PCR™2.1 (Invitrogen) or pGEM-T® Easy (promega), and confirmed by nucleotide sequence analysis (promega). They were then excised using *EcoR*I and used in northern blot analysis as a probe.

Northern blot hybridization. Either twenty micrograms of total RNAs for *Hmg1, Hmg2, Lcy(b)* and *Lcy(e)* or ten micrograms for *Fps, Ggps, Psy, Pds* and *Ccs* was electrophoresed on a 1% agarose/formaldehyde gel, transferred onto nylon membrane and baked for 1 hour at 80°C. To specifically detect mRNAs of *Hmg1* and *Hmg2*, a 210bp *Hpa*I/*Xho*I fragment of HMGR1 and a 530 bp *Bgl*II/*Xho*I fragment of HMGR2 cDNA clones containing 3'-untranslated region were used as a probe. For detecting mRNAs of *Fps, Ggps, Psy, Pds, Lcy(b), Lcy(e)* and *Ccs*, cDNA fragments containing ORF region were used as a probe. Each probe was randomly labelled with α-[^{32}P]-dATP. Hybridization was carried out over-night at 65°C in 0.5M Na_2PO_4 (pH 7.2) buffer, 1% Bovine serum albumin fraction, and 7% SDS. Membranes were rinsed in 2×SSC solution and then washed once at 65°C in 1×SSC solution.

Results and Discussion

Cloning of *Fps, Ggps, Psy, Pds, Lcy(b), Lcy(e),* and *Ccs* cDNAs. Among the genes involved in the isoprenoid biosynthetic pathway, two HMGR genes have been cloned from pepper cDNA library(manuscript in preparation). To further study the molecular regulation mechanism of

Table 1. Summary of PCR strategy for cloning of isoprenoid biosynthetic genes.

Gene	Templates of PCR	DNA sequences of the forward primer/ the reverse primer
Fps	cDNAs of elicitor-treated pepper roots	5'-**ATG**AGTGATTTCAAGTCCAAGTTT-3'/ 3'-TTCTAAATATTCTCCGTCTTC**ATC**-5'
Ggps	genomic DNA of pepper	5'-**ATG**AGATCTATGAACCTTGTTGAT-3'/ 3'-ATATAACGAATAGCACTATTA**ATC**-5'
Psy	mRNAs of elicitor-treated pepper roots	5'-**ATG**TCTGTTGCCTTGTTATGGGTT-3'/ 3'-GAACACGGAAGATGTTCTTGT**ACT**-5'
Pds	mRNAs of elicitor-treated pepper roots	5'-**ATG**CCCCAAATTGGACTTGTTTCTGCT-3'/ 3'-TTCAACCGTCTTTGTTCACATCAA**ATC**-5'
Lcy(b)	mRNAs of elicitor-treated pepper roots	5'-**ATG**GATACGCTCTTGAGAACCCCAAAC-3'/ 3'-TTGTTAAACAATGTCCTATTTCTT**ACT**-5'
Lcy(e)	total RNAs of *Arabidopsis* leaves	5'-**ATG**GAGTGTGTTGGGGC-3'/ 3'-GGATAGAGTTTCAT**ACT**-5'
Ccs	total RNAs of pepper red ripe fruits	5'-**ATG**GAAACCCTTCTAAA-3'/ 3'-GTTATCTCTCGGAA**ACT**-5'

The start and stop codon sequences for gene cloning of ORF size are bolded and underlined.

carotenoid biosynthesis, a branched pathway of plant isoprenoid metabolism, seven genes encoding FPS, GGPS, PSY, PDS, LCY(b), LCY(e) and CCS were cloned using PCR technique (Fig. 2). Oligonucleotides were designed to clone cDNAs containing ORF region of each gene based on the available DNA sequence data of Genbank. *Fps, Ggps, Psy, Pds, Lcy(b)* and *Ccs* were amplified from pepper in sizes of 1031, 1110, 1260, 1749, 1497 and 1497 bp with the accession reference numbers of X84695,[14] X80267,[15] X68017,[13] X68058,[16] X86221[17] and X76165[18], respectively. The cDNA fragment of 1575 bp for *Lcy(e)* was obtained referring to the accession number U50738 from *Arabidopsis*,[19] since pepper *Lcy(e)* is not available at present. For the comparative study of the expression pattern of genes involved in the biosynthesis of carotenoid, northern blot analysis was performed using the above-mentioned genes.

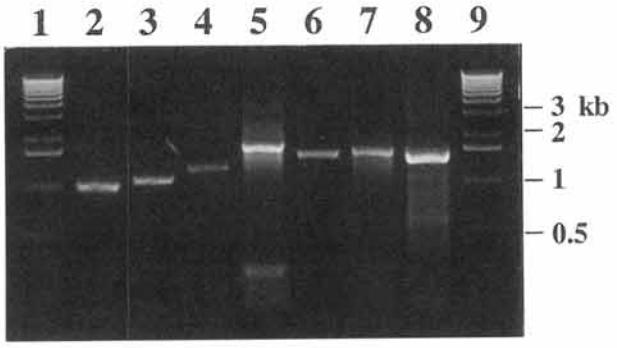

Fig. 2. PCR of seven genes encoding isoprenoid biosynthetic enzymes. RNA templates were derived from elicitor-treated pepper roots for *Psy, Pds* and *Lcy(b), Arabidopsis* leaves for *Lcy(e)* and pepper red ripe friuts (50 DAF) for *Ccs*. *Fps* and *Ggps* were amplified from pepper elicitor-treated roots cDNA and genomic DNA templates. Lane 1 and 9, 1kb ladder; Lane 2, pepper *Fps*, Lane 3, pepper *Ggps*; Lane 4, pepper *Psy*; Lane 5, pepper *Pds*; Lane 6, pepper *Lcy(b)*; Lane 7, *Arabidopsis Lcy (e)*: Lane 8, pepper *Ccs*.

Tissue-specific expression patterns of genes involved in the biosynthesis of isoprenoid in Korean red pepper. As shown in Fig. 3, the tissue-specific expression pattern of nine genes involved in isoprenoid biosynthesis were compared at six tissues containing red ripe fruits (harvested at 50 DAF), mature green fruits (harvested at 35 DAF), leaves, stems, roots, and flowers of pepper. The mRNA level of pepper *Hmg1* was not detected in any tissues. Transcripts of pepper *Hmg2* were present only in stem and root tissues. Two transcripts for *Fps* differing in their size were detected to-gether in stems and leaves. The shorter transcript of *Fps* was detected in all the tissues tested and its signal was stronger in leaves than in others tissues. The mRNA of *Ggps* was detectable in all the tissues except root tissue and its expression was much stronger in leaves and red ripe fruits than in others tissues.

For *Psy*, we also detected two different transcripts based on their size in red ripe fruits. The longer transcript (*Psy1*) was highly expressed only in flowers and ripening fruit tissues containing chromoplast while the shorter one (*Psy2*) was expressed in all tissues except flowers and in particular, its higher level was detected in leaves as Bartley *et al.* previously discussed from tomato.[9,20] The presence of two different transcripts for *Pds* was also confirmed from the leaves as well as red ripe fruits of Korean red pepper while only single transcript of *Pds* (ca. 2.0 kb) was detected in seedling, leaves and fruits of bell pepper, which are even within same species, in previous report by Hugueney *et al.*.[16] The shorter transcript for *Pds* was presumably expressed in all green tissues and also expressed in chromoplast-containing tissues of red ripe fruits and flowers. Unlike a chromoplast-specific tomato *Pds* expressed only in ripening fruits and flowers,[21,22] chromoplast-specific *Pds* was not identified in Korean red pepper. *Lcy(b)* and *Lcy(e)* genes involved in biosynthesis of the β- and α-carotene. Relatively, lower levels of transcrips for *Lcy(b)* and *Lcy(e)* were exhibited in all the tissues, but

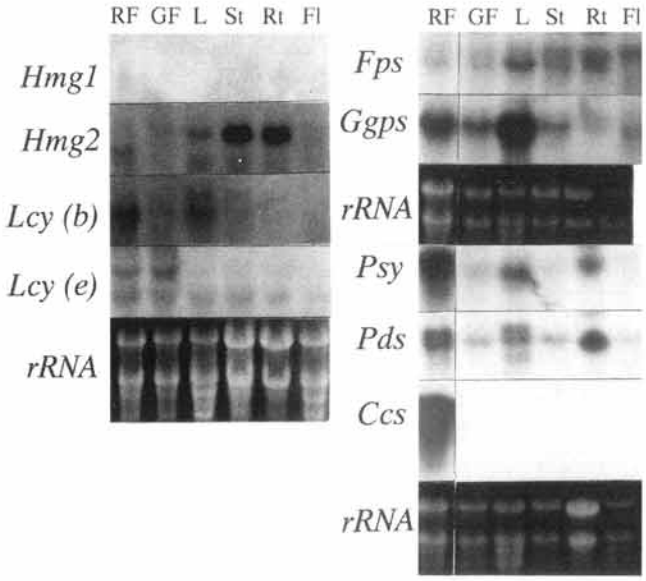

Fig. 3. Tissue specific expression of *Hmg1*, *Hmg2*, *Fps*, *Ggps*, *Psy*, *Pds*, *Lcy(b)*, *Lcy(e)*, and *Ccs*. Twenty micrograms for *Hmg1*, *Hmg2*, *Lcy(b)* and *Lcy(e)* and ten micrograms for *Fps*, *Ggps*, *Psy*, *Pds* and *Ccs* of total RNAs derived from several tissues were blot hybridized with the ^{32}P-labeled gene specific probes for *Hmg1* and *Hmg2* and cDNA probes for *Fps*, *Ggps*, *Psy*, *Pds*, *Lcy(b)*, *Lcy(e)* and *Ccs*. RF: red fruit (50 DAF), GF: green fruit (35 DAF), L: leaf, St: stem, Rt: Root and Fl: flower.

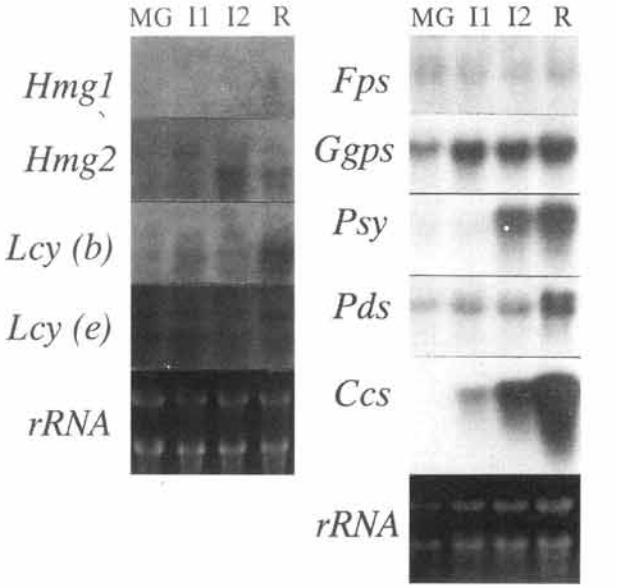

Fig. 4. The expression of *Hmg1*, *Hmg2*, *Fps*, *Ggps*, *Psy*, *Pds*, *Lcy(b)*, *Lcy(e)*, and *Ccs* during ripening of pepper fruits. Total RNAs were derived from pepper fruits at four different ripening stages. Twenty micrograms of total RNAs for *Hmg1*, *Hmg2*, *Lcy(b)* and *Lcy(e)* and ten micrograms of total RNAs for *Fps*, *Ggps*, *Psy*, *Pds* and *Ccs* were used. The probes used in this experiment were the same as described in Fig. 3. MG: mature green stage (fruits at 35 DAF), I1: intermediate ripe stage 1 (fruits at 40~45 DAF), I2: intermediate ripe stage 2 (fruits at 45~50 DAF), and R: red ripe stage (fruits at 50 DAF).

Lcy(b) was more or less strongly expressed than *Lcy(e)* in red fruits and leaves. *Ccs* which is responsible for the red colour of pepper fruits, was expressed only in fruits during the ripening stages and not expressed in any other tissues tested including green fruits.

Comparison of the expression of genes involved in the biosynthesis of isoprenoid during pepper fruit ripening. Fruits of Korean red pepper were divided into four groups from mature green to the full red colour and northern blot analysis of nine different genes was carried out to observe their expression pattern of ripening stages (Fig. 4). The transcripts of *Hmg1* and *Hmg2* were not present in all stages of fruits ripening. *Fps* was constitutively expressed during fruit ripening and might not play a critical role in the regulation of carotenoid biosynthesis as expected. In the mature green pepper, all mRNAs of *Ggps*, *Psy* and *Pds* were observed as basal level and they were induced in a sequence manner during ripening. The expression of *Ggps* showed the highest levels at the early ripening stage (Intermediate ripe stage 1 of Fig. 4) and its mRNA level was slightly decreased during fruit ripening. The expression of *Psy* started to be induced strongly in semi-ripening stage (Intermediate ripe stage 2 of Fig. 4), *Pds* mRNA was maximally induced in the fully ripened stage (Red ripe stage of Fig. 4) unlike both *Psy* and *Pds* of tomato were expressed as the greastest levels at intermediate orange stage of fruit.[23] Both the shorter and longer transcrips of *Psy* and *Pds* were detected together at inducing point of each gene during fruit ripening. The mRNA level of *Ccs* was detectable not in green fruit stage but only during the ripening period (I1~R lanes of Fig. 4).

In summary, the expression of *Hmg1*, *Hmg2* and *Fps* might be not so critical in pepper carotenoid biosynthesis and the other six genes of *Ggps*, *Psy*, *Pds*, *Lcy(b)*, *Lcy(e)* and *Ccs* were coordinately regulated during ripening of pepper fruit because these transcripts became detectable in accordance with the accumulation of carotenoids. These results are different from those observed by Romer *et al.* describing that the genes encoding GGPS, PSY and PDS were not co-regulated during fruit ripening of bell pepper.[13] In detail, *Psy* mRNA are barely detectable and only the *Pds* mRNA is present at the mature green. The *Ggps* mRNA is first detected at early ripening stage and *Psy* mRNA remains clearly lower than of *Ggps* mRNA, even in later ripening stage. Here we reveal that at least three genes encoding GGPS, PSY and PDS were induced in a sequential manner according to ripening degree and are certainly co-regulated for carotenoid accumulation in Korean red pepper.

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