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3-Hydroxy-3-methylglutaryl coenzyme A reductase 1 (*HMG1*) is highly associated with the cell division during the early stage of fruit development which determines the final fruit size in *Litchi chinensis*

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ABSTRACT

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC: 1.1.1.34), an enzyme catalyzing the first committed step in the mevalonic acid (MVA) pathway for the biosynthesis of isoprenoids, has been reported to be involved in the fruit size determination through the regulation of early cell division. In litchi, the cell number achieved by this early cell division determines the final fruit size, but whether HMGR plays any role in this process was unknown. In this study, we set out to address this question with gene cloning and expression analysis in fruits of different pheno- or genotypes. We found that the litchi genome includes two HMGR homologues, denoted as *LcHMG1* and *LcHMG2*. Despite 70% sequence identity at the amino acid level, they exhibited distinct expression patterns during litchi fruit development. *LcHMG1* expression was highest in the early stage of fruit development, correlated with the high level of cell division. Absolute levels of *LcHMG1* expression varied among fruits of different pheno- or genotypes, with expression in large-fruited types reaching higher levels for longer duration compared to that in small-fruited types. The expression patterns for *LcHMG1* strongly suggest that this gene is involved in early cell division and fruit size determination in litchi. In contrast, *LcHMG2* was most highly expressed in the late stage of fruit development, in association with biosynthesis of isoprenoid compounds required for later cell enlargement. These findings provided new insights on the function of HMGR genes during fruit development.

1. Introduction

Fruit size is an essential parameter for growers, since it will determine if the fruit goes to the fresh market for a good profit or to the processing factory for less. The fruit size is determined not only by the cell number defined during early cell division stage after fertilization, but also by cell volume achieved through the afterward cell enlargement (Bohner and Bangerth, 1988; Cowan et al., 2001; Narita and Gruissem, 1989). Any factors affecting the cell division and cell enlargement, such as genetic characteristics, phytohormones, nutrients, and culture practices, will contribute to the final fruit-size determination. In addition to these, the time of anthesis is an important factor in many fruits, including apple (Marguery and Sangwan, 1993), kiwifruit (Cruz-Castillo et al., 2002; Lai et al., 1990), peach (Scorza et al., 1991), grape (Coombe, 1973), citrus (Praloran et al., 1981), strawberry (Cheng and Breen, 1992) and litchi (Li et al., 2004). In general, flower buds of early anthesis within the same tree tend to give rise to larger fruits at maturity compared to those of later anthesis. This phenomenon always leads to the lack of uniform fruit size at harvest and therefore compromises the fruit quality and its commercial value.

Litchi (*Litchi chinensis*) is one of the most valuable commercial fruits in the subtropical area, and its fruit size is of important commercial value. Many factors that affect litchi fruit size, such as genetic characteristics, pericarp development, cytology, temperatures and water regime, phytohormones, nutrients, plant regulators, pruning and girdling, have been extensively analyzed and documented (Li et al., 2010). The 'Feizixiao' litchi, a popularly cultivated cultivar in southern China, Australia and Thailand, is prone to unsynchronized flowering, which causes the production of fruits with substantially uneven fruit sizes (Li et al., 2004). As is true for most other fruits, the final litchi fruit size is determined by cell number rather than cell volume (Li et al., 2002). Hence, cell division as well as factors that affect cell division could play a crucial role in regulation of fruit size in litchi. One of such factors could be the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC: 1.1.1.34), which has shown to affect fruit size in

Abbreviations: EA, Early anthesis; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; LA, Late anthesis; MEP, methyl-D-erythritol-4-phosphate; MVA, mevalonate; ORF, Open reading region; qPCR, Quantitative PCR; RACE, Rapid amplification of cDNA ends; UTR, Untranslated region.

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several species (Cowan et al., 2001; Kobayashi et al., 2003; Narita and Gruissem, 1989).

The implication of the role of HGMR in regulation of fruit size was firstly demonstrated in tomato by Narita and Gruissem (1989), who proposed that tomato HMGR was required early in fruit development but not during ripening. HMGR genes, typically encoded by a small multigene family in plants, have been isolated from many plant species. For example, two distinct genes have been isolated and characterized in Arabidopsis (Caelles et al., 1989; Enjuto et al., 1994), tomato (Narita and Gruissem, 1989; Rodriguez-Concepcion and Gruissem, 1999), cotton (Loguercio et al., 1999), apple (Rupasinghe et al., 2001), pear (Cong et al., 2009) and longan (Xia et al., 2011); three in potato (Choi et al., 1992) and even a larger family in rubber (Chye et al., 1992; Sando et al., 2008), rice (Nelson et al., 1994), corn (Stermer et al., 1994) and legume (Kevei et al., 2007). HMGR catalyzes the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate (MVA), the first committed step in cytosolic isoprenoid biosynthesis (Caelles et al., 1989; Chappell et al., 1995; Goldstein and Brown, 1990). The reaction is irreversible and is also considered to be the rate-limiting step in isopentenyl pyrophosphate (IPP) biosynthesis (Chappell et al., 1995). Isoprenoids, a large and diverse group of compounds widespread in eukaryotes, constitute major vital compounds that are involved in a wide range of primary and secondary metabolism pathways in plants, and production of essential substances, including growth regulators (abscisic acid, gibberellins, and cytokinins), chlorophylls and plastoquinone in photosynthesis, carotenoids in photo-protection, and a few phytoalexins and steroids required for the assembly of biological membranes(Cowan et al., 1997; Lange et al., 2000; Stermer et al., 1994). Although the isoprenoid biosynthesis in plants consists of two pathways (long-known mevalonate (MVA) pathway and more recently discovered methyl-D-erythritol-4-phosphate (MEP) pathway), which operate independently of each other by virtue of being localized to separate intracellular compartments (Chappell, 2002; Rodriguez-Concepcion and Boronat, 2002; Rohmer et al., 1996), the existence of frequent exchanges and complex crosstalk make them operate synergistically in plants (Eisenreich et al., 2001; Rodriguez-Concepcion et al., 2004). MVA-derived precursors can be used for the synthesis of isoprenoids in the plastid, while MEP-derived precursors can be exported to the cytosol in certain plants, tissues, and developmental stages (Eisenreich et al., 2001).

Rodriguez-Concepcion and Gruissem (1999) further demonstrated a close correlation between *HMG1* expression and tomato fruit growth, strongly suggesting that a major function of *HMG1* is to ensure adequate phytosterol production for cell division and cell enlargement. Using avocado as a model system, Cowan et al. (2001) declared that HMGR was clearly central in the metabolic control of fruit growth. Activity of HMGR modulates availability of regulatory isoprenoid products required for cell division, sink strength and fruit growth (Cowan et al., 2001). High levels of HMGR activity and expression were always observed in meristematic tissues and suspension cultured cells active in cell division and growth (Aoyagi et al., 1993; Cowan et al., 1997; Enjuto et al., 1994; Kato-Emori et al., 2001; Rupasinghe et al., 2001; Xia et al., 2011). However, the most direct evidence that reveals HMGR as an important fruit size regulator was demonstrated in transgenic plants. Over-expression of a melon HGMR gene in tomato substantially stimulated cell division activity and resulted in an increase of the cell layers in the fruit pericarp, consequently leading to the production of much larger fruit in transgenic plants compared to the fruit in wild-type plants (Kobayashi et al., 2003).

In this study, we isolated and characterized two litchi HMGR homologues and performed detailed expression analyses in various tissues and fruits of different pheno- or genotypes during fruit development, aiming at elucidating the role of litchi HMGR genes in regulation of cell division activity and fruit size. Our results show that two litchi HGMR genes are differentially regulated during litchi fruit development, and the expression of litchi *HMG1* is highly correlated with cell division activity in young litchi fruit. The possible role of these two HMGR genes involved in the fruit size determination is discussed.

2. Materials and methods

2.1. Plant materials

Materials for gene cloning were collected from three 'Ziniangxi' litchi trees in an orchard located at Dongguan Institute of Agricultural Science in 2005 and 2006. The other experimental materials were all harvested in an orchard located at South China Agricultural University, Guangzhou, China in 2009.

Three early-anthesis (EA, on March 20) and three late-anthesis (LA, on April 10) 'Feizixiao' trees, and three 'Silianggguo' (large-fruit genotype) and three 'Chenzi' (small-fruit genotype) trees were selected to compare their cell division activities and gene expressions. Each tree was treated as a biological replicate. Twenty panicles located in different directions of each tree were tagged. Ten of them were used for tracing the diameters of marked fruits, and the other ten were for periodical fruit sampling. Ten fruits from ten panicles having the similar size to the labeled ones were collected and weighted at each sampling date. The collected fruits were placed in an icebox immediately before dissection into pericarp, aril and seed tissues, after which tissues were stored at −80 °C until use. Samples for different organs and tissues were collected from the same 'Hehuadahongli' litchi tree.

2.2. Total RNA preparation and cDNA cloning

Total RNA was isolated from approx 10 g of frozen tissue using the hot borate method (Lu and Jiang, 2003; Wan and Wilkins, 1994). RNA quality was determined by denaturing agarose gel [1.2% (w/v) agarose, 0.5× MOPS] and spectrophotometer. The full-length HMGR cDNA was cloned by RT-PCR, in combination with 3′- and 5′-RACE using degenerate or specific primers (Table S1). RT-PCR was carried out following a two-step procedure. First strand cDNA was synthesized using AMV Reverse Transcriptase XL (TaKaRa, Daliang, P. R. China) and oligo$(dT)_{18}$ primer. PCR amplifications were performed using *Ex Taq* polymerase (TaKaRa, Daling, P. R. China) under different annealing temperatures, extension times and cycles according to the melting point of primer pairs (Table S1) and the expected size of DNA fragments to be amplified. The PCR products were cloned into the pMD 18-T vector (TaKaRa). Their sequences were determined (Invitrogen, Shanghai, P. R. China) and searched against the Genbank database at the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) for homology analysis. Gene-specific primers (Table S1) of each gene fragment were then designed and used for 3′-RACE (Rapid amplification of cDNA end; 3′-Full RACE Core Set Ver.2.0; TaKaRa) according to the manufacturer's instructions. 5′-RACE was carried out using a modified TDT (terminal deoxynucleotidyl transferase) tailing method (Xia et al., 2008). cDNA was obtained by reverse transcription using a short gene-specific primer. A nested-PCR strategy was used for the amplification of desired 5′ terminal sequences. The products obtained from 3′- and 5′-RACE were cloned and determined as described above.

2.3. Sequence analysis

Full-length cDNA sequence of *LcHMG1* and *LcHMG2* was assembled through sequential ligation and cloning, and verified by DNA sequencing. Sequence homology was analyzed by BLAST-algorithm. Multiple sequence alignment analysis was performed using ClustalW (http://www.ebi.ac.uk/clustalw/). The alignment was visualized using the Boxshade v3.21 software (http://www.ch.embnet.org/

software/BOX_form.html). A phylogenetic tree was constructed by the neighbor-joining method based on the Phylip program (http://bioweb.pasteur.fr/phylogeny/intro-en.html).

2.4. Flow cytometric analysis

DNA content was estimated using flow cytometry according to Li et al. (2011). In brief, fruit samples were ground gently in ice cold Otto buffer I containing 100 mmol·L^{-1} citric acid (Otto, 1990). In this study, Otto I and Otto II buffers were mixed in a 1:2 ratio. The buffers were supplemented with 30 mmol·L^{-1} DTT, 10% (w/v) PVP, 2% (v/v) Triton X-100, 50 mmol·L^{-1} β-mercaptoethanol and 100 μg·mL^{-1} RNase A for restraining the activity of hydroxybenzene. Nuclear suspension was strained with 50 μg·mL^{-1} propidium iodide (PI) and incubated for 30 min in the dark. Strained nuclei were filtered through a nylon filter (pore size, 38 μm) and analyzed using a FACS Calibur (Becton-Dickinson, USA) flow cytometric (FCM).

Cell division activity was denoted by the proportion of cells involved in division, which were estimated as the ratio of the nuclei within the S and G2/M phases to the total number of nuclei within the S, G2/M and G0/G1 phases. The cell phase was determined by the FCM data using WinMDI software.

I.e., Cell division activity

$$= \left(N_{G2/M} + N_S\right) / \left(N_{G0/G1} + N_{G2/M} + N_S\right) \times 100\%.$$

$N_{G2/M}$, Ns and $N_{G0/G1}$ were the number of nuclei within G2/M, S and G0/G1 phases, respectively.

2.5. Real-time quantitative RT-PCR

To facilitate the real-time PCR analysis of all investigated genes under the same reaction conditions, specific primers were designed using Primer Premier 5.0 software under default parameters, and synthesized by a commercial supplier (Sangon, Guangzhou, P. R. China). Regular RT-PCR amplification was performed to check the specificity of all primer pairs, and products of desired size were analyzed on a 1.2% agarose gel and sequenced by Invitrogen (Shanghai, P. R. China) to confirm the sequences of the amplicons. The effectiveness of primer pairs was further confirmed with the melting-curve analysis. No further analysis was performed on primers that had multiple products or products in no template control (NTC). Standard curve using a dilution series of a mixed cDNA (spanning six orders of magnitude) was made to calculate the gene-specific PCR efficiency and regression coefficient (R^2) for each gene.

Two micrograms of total RNA was used to synthesize cDNA in a 20 μL reaction volume using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed using Power SYBR Green PCR Master Mix Kit (Applied Biosystems) on an Applied Biosystems 7500 Real-time PCR System according to the manufacturer's instructions. Real-time samples were run in triplicate for each biological replicate and the reaction volumes were 20 μL, which contains 10 μL of SYBR Green PCR Master Mix (Invitrogen), 2 μL of each primer, 60 ng cDNA template and 0.1 μL ROX. The qPCR was subjected to the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s in 96-well optical reaction plates (Bio-Rad, USA). Dissociation curves were run to determine the specificity of the amplification reactions. After validation tests, normalization to actin was performed using the $\Delta\Delta C^T$ method (Applied Biosystems). The gene characteristics and their primer sequences were listed in Table S2.

3. Results

3.1. Isolation and characterization of litchi HMGR homologues, LcHMG1 and LcHMG2

To isolate litchi HMGR homologues, several degenerate primer pairs were designed and used for RT-PCR amplification of litchi cDNA samples. Initially, two partial cDNA fragments representing two distinct homologues were isolated and designated as *LcHMG1* and *LcHMG2*, respectively. Full length of two cDNA sequences was obtained and assembled by 5′ and 3′ RACE analysis. The resulting full-length *LcHMG1* cDNA (2256 bp in length), comprises a 75 bp 5′ untranslated region (5′-UTR), a 1689 bp open reading frame (ORF), and a 492 bp 3′ untranslated region (3′-UTR). It encodes a putative polypeptide of 562 amino acids. Similarly *LcHMG2* cDNA (2209 bp in length), which contains a 154 bp 5′-UTR, a 1707 bp ORF and a 348 bp 3′-UTR, encodes a putative polypeptide of 568 amino acids, which is six amino acids longer than LcHMG1. BLAST search against GenBank showed that LcHMG1 shared over 75% sequence identity with HMGR proteins from many other species, and exhibited the highest level of identity (81%) with *Ricinus communis* HMGR (XP_002510732), and LcHMG2 shared the highest level of identity (74%) with the *Gossypium hirsutum* HMG2 (O64967). Sequence alignment between LcHMG1 and LcHMG2 revealed an approximate 77% sequence identity, indicating substantial sequence divergence during evolution.

LcHMG1 and LcHMG2 share typical domain features identified in other plant HGMRs, including the defined N-terminal region, membrane domain, linker region, and catalytic domain (Fig. S1). Among them the membrane and the catalytic domains were well conserved, whereas the N-terminal and the linker regions were highly divergent. The existence of two highly conserved hydrophobic sequences (H1 and H2) in the membrane domain suggested that these two HMGRs might target to a membrane system. In the catalytic domain two HMG-CoA binding motifs [EMPV(I)GYVQV(I)P and TTEGCLVA] and two NADPH-binding motifs (DAMGMNM and GTVGGGT) were highly conserved. The amino-terminal of all the HMGRs also contained the conserved motif MD/EXRRR [MetAsp/GluXArgArgArg (X can be Val, Leu, and Pro)]. These results strongly suggest that both LcHMG1 and LcHMG2 should play a role in cytosolic isoprenoid biosynthesis.

Phylogenetic tree analysis (Fig. S2) revealed that HMGRs from each taxon had its own characteristics distinct from other HMGRs. Angiosperms clustered into a separate clan as did Gymnosperms, and the Angiosperm clan was further divided into dicotyledon and monocotyledon subclans. LcHMG1 and LcHM2, which were firstly clustered together with their counterparts from longan DlHMG1 and DlHMG2, respectively (Xia et al., 2011), were distributed into distinct subclans in dicotyledons. There were other subclans apart from the two containing LcHMG1 and LcHMG2, consistent with the fact that many plants contain more than two HMGR homologues.

3.2. Fruit development in fruits of different pheno- or genotypes

To explore the mechanism of fruit size determination in litchi, we chose two kinds of fruits with different phenotypes (fruits of different final fruit sizes in a given cultivar) and two with different genotypes (fruits from different cultivars) for contrasting studies. Fruits of different phenotypes were collected from the cultivar 'Feizixiao', which is prone to have unsynchronized flowering every year rendering the production of large fruit from early anthesis (EA) and small fruit from late anthesis (LA). And 'Silliangguo' and 'Chenzi', were selected as representatives of large- and small-fruit genotypes, respectively.

Firstly fruit development patterns in fruits of different pheno- and genotypes were investigated. As far as two phenotypes are concerned, both fruits follow the same single sigmoid pattern with two distinct stages (Fig. 1A). The stage I for EA and LA fruits is characterized by the

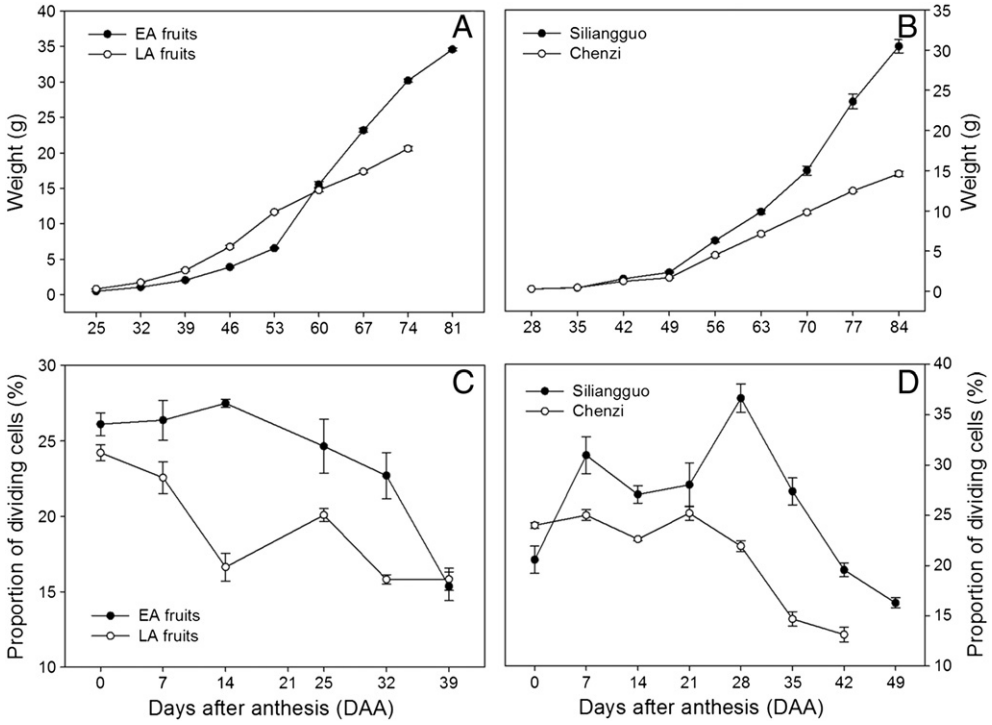

Fig. 1. Fruit development and cell division activity in fruitlet or pericarp of large- and small-fruits of different genotypes (B and D) and phenotypes (A and C).

rapid cell division and lasts till 46 DAA and 39 DAA, respectively. At the end of stage I the fruit weight of EA fruits was comparable to that of LA fruits. However, these fruits display size differentiation at stage II and this specifically occurs on or after 60 DAA and 53 DAA for EA and LA fruits, respectively, due to rapid development of the aril during this period. The final mean fruit weight of EA fruits was about 34.58 g, while LA fruits were only 20.58 g. As for different genotypes, 'Siliangguo' bore large fruits of 30.47 g on average while 'Chenzi' produced small fruits of only 14.61 g on average (Fig. 1B). A similar single sigmoid development pattern was observed in the fruits from both genotypes. Their stage I lasted till 49 DAA, after which the fruit began to enlarge rapidly.

3.3. Cell division activity analysis in fruits of different pheno- or genotypes

In tomato and avocado, the final fruit size is determined by cell number achieved by early cell division (Cowan et al., 1997, 2001; Gillaspy et al., 1993; Narita and Gruissem, 1989). To determine if a parallel relationship between early cell division and final fruit size is evident in litchi, the cell division activity in fruits of different pheno- or genotypes was characterized by flow cytometry. The proportion of cells involved in each division phase was used to denote the cell division activity. Since cell division mainly occurred in the early stage of fruit development, only young fruits (fruits in stage I) were selected for this analysis. The EA fruits, the large-fruit phenotype, possessed a significantly higher cell division activity than the LA fruits, the small-fruit phenotype, during the entire early developmental course analyzed (Fig. 1C). Cell division activity in the EA fruits was maintained constantly above 25% before 14 DAA, and decreased gradually afterward. However, the cell division activity in the LA fruits dropped substantially from approximately 24% at 0 DAA to 17% at 14 DAA and recovered a little thereafter. Consistently, differential cell division activity in young fruits was also observed between the large-fruit genotype 'Siliangguo' and the small-fruit genotype 'Chenzi'. The cell division rate in 'Siliangguo' was higher than that of 'Chenzi' during the entire early fruit development except 0 DAA (Fig. 1D). 'Siliangguo' showed a rapid increase and persistence of high division rate until 35 DAA, followed by a steep drop. Two cell division peaks were achieved in 'Siliangguo' at 7 DAA and 28 DAA, while the cell division in 'Chenzi' maintained a steady level (25%) before 21 DAA and decreased quickly thereafter.

Taken together, these results indicate that young fruits from the larger-fruit phenotype (EA fruits) or genotype ('Siliangguo') were more active in cell division and maintained a longer period of high division rate during the entire early fruit developmental stage compared to those from the small-fruit phenotype (LA fruits) or genotype ('Chenzi'), confirming that high cell division activity in early stage of litchi fruit development is a key factor in determination of final fruit size, as reported in tomato and melon.

3.4. Analysis of LcHMG1 and LcHMG2 expressions in various litchi tissues, developmental stages and genotypes

High expression of HMGR genes in tomato and melon correlates with their high cell division activity in the early stage of fruit development. To test whether this correlation exists in litchi and explore the possible role of *LcHMG1* and *LcHMG2* in regulation of cell division and litchi fruit size, we analyzed *LcHMG1* and *LcHMG2* expressions in various tissues and during fruit development in different pheno- or genotypes. Fig. 2 shows that the expression of *LcHMG1* and *LcHMG2* was universally detected in all the tissues analyzed, including unfertilized female flowers, fruit tissues, leaves, shoots, and roots but their expression level appeared to vary from tissue to tissue, with the highest expression found in the young pericarp (39 DAA) and the lowest in the aged seed (81 DAA). In general, *LcHMG1* displayed a high level of expression in young tissues compared to aged tissues for a given organ except root, with this pattern more noticeable for the fruit tissues (pericarp, aril, and seed), while *LcHMG2* was very active in young (e.g. young pericarp and seed) as well as aged tissues (e.g. aged aril and leaf). It is noted that the *LcHMG2* transcripts in aged aril (81 DAA) was approximately ten times more abundant than that in young aril (39 DAA).

Expression of *LcHMG1* and *LcHMG2* appears to be differentially regulated in fruits of different pheno- or genotypes. The transcription

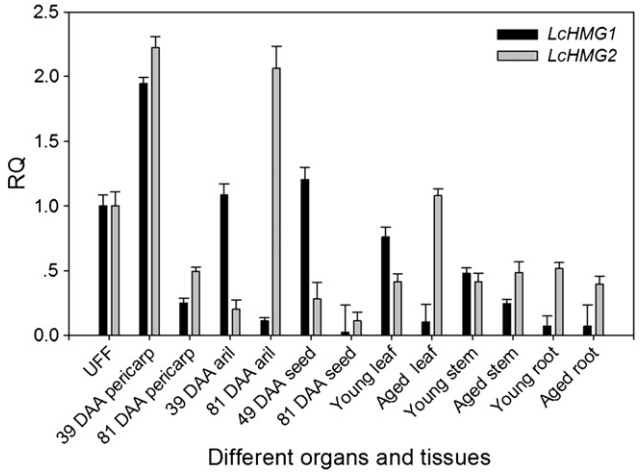

Fig. 2. Expression analysis of *LcHMG1* and *LcHMG2* in various litchi organs and tissues. The expression levels of *LcHMG1* and *LcHMG2* were normalized using actin. Data are means ± SE (n = 3). The values of *LcHMG1* and *LcHMG2* from UFF (unfertilized female flower) were arbitrarily set to 1. RQ: relative quantitation.

activity of *LcHMG1* peaked in the early stage and gradually decreased along with the fruit development. However, the abundance of *LcHMG1* transcripts varied in different fruits. Fruits of large-fruit pheno- or genotypes tended to accumulate relatively more *LcHMG1* mRNAs than those of small-fruit pheno- or genotypes, especially during the early stage of fruit development. In the pericarp, the *LcHMG1* transcript level in the EA fruits exhibited peaks at 0, 14 and 39 DAA and its high-level expression lasted until 53 DAA, while it attained only one peak at 0 DAA in the LA fruits and dropped dramatically after 39 DAA (Fig. 3A). In the aril, *LcHMG1* transcripts before 60 DAA were much more abundant in the EA fruits than in the LA fruits in the same period (Fig. 3B). During seed development, *LcHMG1* maintained high-level expression in both EA and LA fruits but started declining at a much earlier time (32 DAA) in LA fruits compared to EA fruits (39 DAA) (Fig. 3C). As far as fruits from different genotypes were concerned, *LcHMG1* showed a much higher expression in pericarp of 'Siliangguo' than that observed in the same tissue of 'Chenzi' before 49 DAA except for 35 DAA (Fig. 4A). The expression level of *LcHMG1* in the aril was comparable in the two types of fruits although a slightly higher level was detected in 'Siliangguo' (Fig. 4B). In the seed, *LcHMG1* showed a higher level of expression in 'Siliangguo' than in 'Chenzi' before 49 DAA (Fig. 4C). As a whole, the large fruits ('Siliangguo' and EA fruits), compared to the small fruits ('Chenzi' and LA fruits), not only attained a substantially higher level of *LcHMG1* expression in the early stage, but also maintained a longer period of this high-level expression in all fruit tissues investigated. This expression pattern was highly correlated with the cell division activity detected in the fruit, implying a possible direct relationship between *LcHMG1* expression and cell division activity.

The expression pattern of *LcHMG2* varied greatly among the three fruit tissues although comparable patterns within a given tissue of different pheno- or genotypes were observed. *LcHMG2* transcription in the pericarp peaked at 7 DAA from a low-level at the full-bloom (0 DAA) in both EA and LA fruits but this peak was much more pronounced in LA fruits (Fig. 3D). In the aril, *LcHMG2* expression was up-regulated in the late stage of development as evidenced by a sharp rise at 53 DAA and 60 DAA in LA fruits and EA fruits, respectively (Fig. 3E). This pattern is in contrast to the *LcHMG1* expression pattern observed in the same tissue of EA and LA fruits (Fig. 3B). Similarly, a distinct expression pattern was also observed between *LcHMG1* and *LcHMG2* during seed development, where *LcHMG2* showed a peak transcription at 53 DAA in the LA fruits and 60 DAA in the EA fruits (Fig. 3F) instead of a peak at the beginning followed by a gradual decline as observed for *LcHMG1* (Fig. 3C). As for fruits of different genotypes, 'Siliangguo' gave rise to a consistent and high level of *LcHMG2* expression throughout the entire pericarp development compared to that observed in 'Chenzi' (Fig. 4D), but the latter genotype displayed more robust LcHMG2 transcriptional activity after 70 DAA during the aril development relative to 'Siliangguo' (Fig. 4E). During seed development, *LcHMG2* expression in both genotypes dropped to the lowest level at 49 DAA and rebounded after that, but 'Chenzi' showed stronger *LcHMG2* expression before 49 DAA while 'Siliangguo' displayed stronger rebound after 49 DAA (Fig. 4F). Taken together, the expression pattern of *LcHMG2* is distinct from that of *LcHMG1* in all fruit tissues regardless of pheno- or genotypes. It was also noted that *LcHMG2* was highly expressed in the late

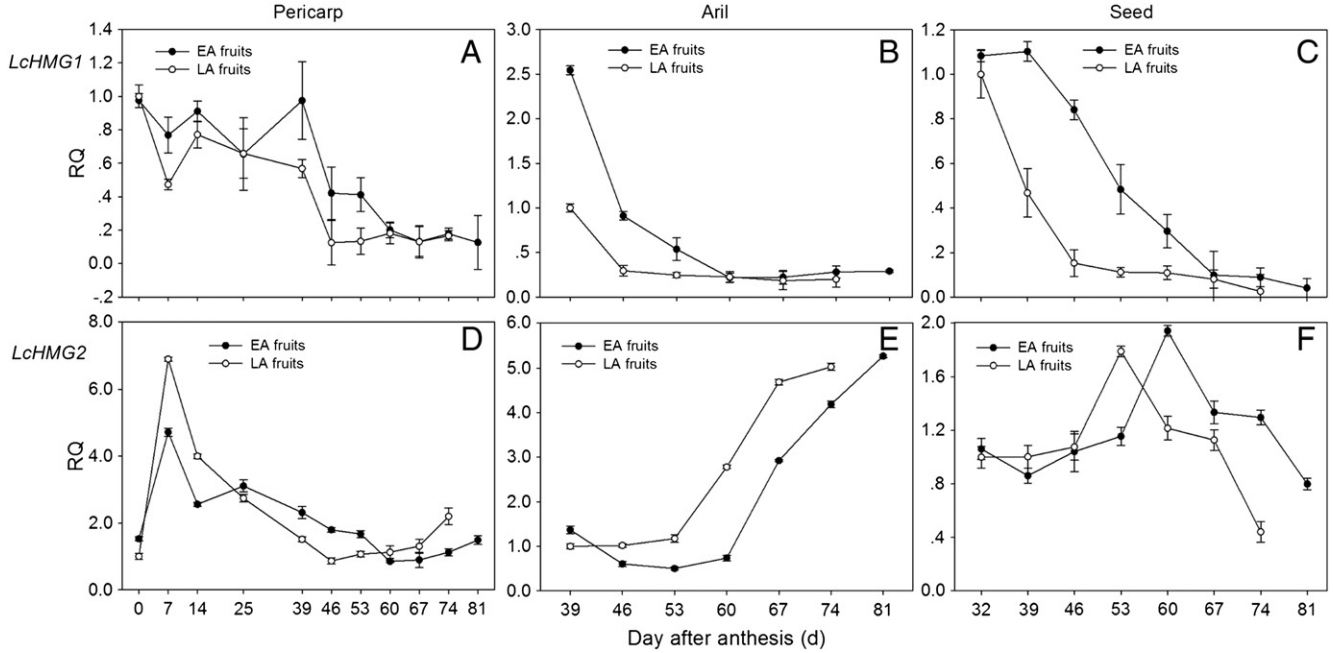

Fig. 3. Expression analysis of *LcHMG1* and *LcHMG2* during fruit development in fruits of different phenotypes. The expression levels of *LcHMG1* and *LcHMG2* were normalized using actin. Data are means ± SE (n = 3). The values of *LcHMG1* and *LcHMG2* from LA fruits at the first time point were arbitrarily set to 1. RQ: relative quantitation.

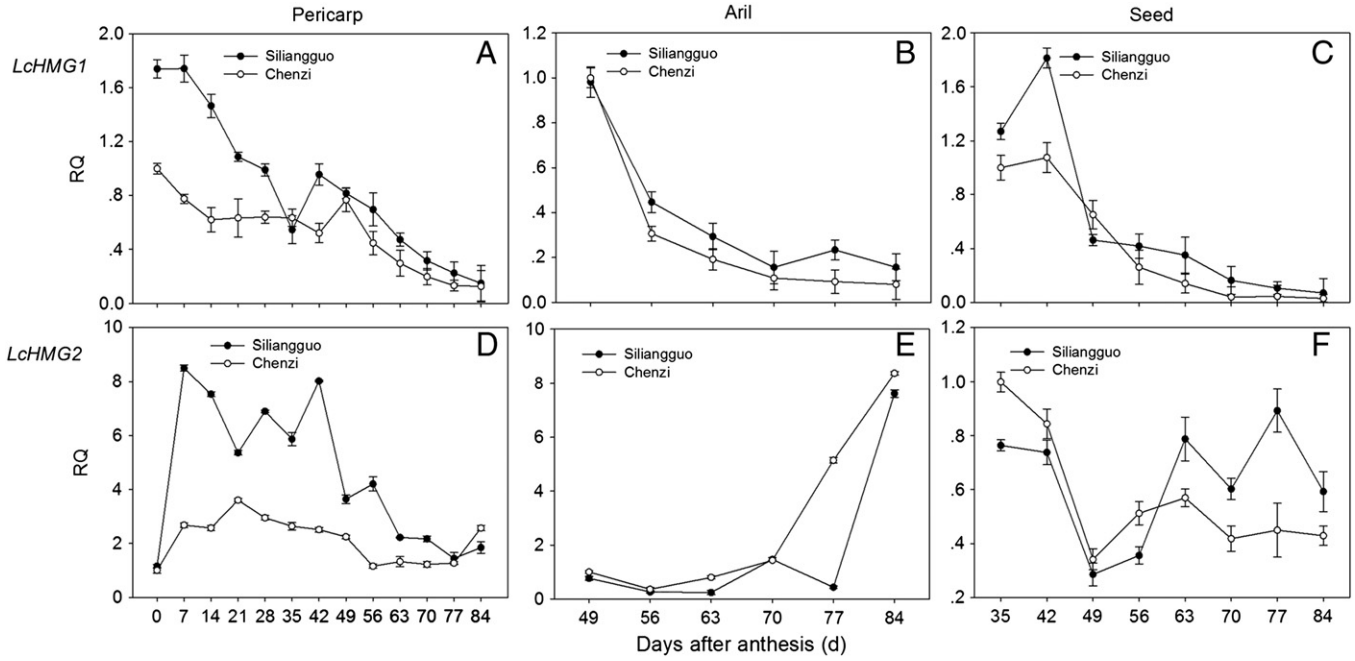

Fig. 4. Expression analysis of *LcHMG1* and *LcHMG2* during fruit development in fruits of different genotypes. The expression levels of *LcHMG1* and *LcHMG2* were normalized using actin. Data are means ± SE (n = 3). The values of *LcHMG1* and *LcHMG2* from 'Chenzi' fruits at the first time point were arbitrarily set to 1. RQ: relative quantitation.

stage of aril development in contrast to the high level of *LcHMG1* expression during early aril development.

4. Discussion

4.1. Two HMGR homologs have been isolated from litchi fruits

HMGR activity and function are highly conserved in plants, and the biological properties of the enzyme have been extensively characterized. Plant HMGRs comprised two conserved domains (membrane domain and catalytic domain) and two divergent regions (N-terminal region and linker region) (Campos and Boronat, 1995; Monfar et al., 1990; Rupasinghe et al., 2001). In contrast to HMGRs from other organisms which have seven trans-membrane regions, plant HMGRs contain two membrane-spanning regions, corresponding two hydrophobic segments in the membrane domain (Caelles et al., 1989; Campos and Boronat, 1995).The active substrate-binding motifs (HMG-CoA and NADPH binding motifs) in the catalytic domain of human HMGR are extremely conserved in plant HMGRs as well (Istvan et al., 2000; Liao et al., 2004; Xia et al., 2011). HGMRs have been proved to be essential for fruit development. To date, HMGR genes have been cloned from considerable horticultural fruit crops (Choi et al., 1992; Cong et al., 2009; Kobayashi et al., 2003; Narita and Gruissem, 1989; Rupasinghe et al., 2001; Xia et al., 2011). In the present work we isolated two HGMR homologs from litchi, *LcHMG1* and *LcHMG2*, which shared great identities with each other and with their orthologs from other plants (Fig. S1). They possess all the properties conserved among plant HMGRs, which strongly suggest that these two HMGRs should be functional proteins catalyzing the production of mevalonate.

4.2. LcHMG1 transcription activity is highly correlated with cell division activity during litchi fruit development

In most plants, fruit development can be divided into two distinct phases: cell division and cell enlargement (Gillaspy et al., 1993). Rapid cell division, proceeded with the successful pollination and fertilization, always occurs at the early stage of fruit development (Gillaspy et al., 1993; Kobayashi et al., 2002). Litchi fruit growth was found to follow a similar two-stage growth pattern. Stage I (0–53 DAA) is characterized by the rapid growth of pericarp and seed coat with active cell division, and stage II (53–88 DAA) is dominated by the growth of embryo and aril with rapid cell enlargement (Li et al., 2003a). Huang and Xu (1983) proposed a "ball-skin versus bladder effect" to conceptualize the restraints exerted by a pre-formed fruit skin to the latter expanding aril. The weight of the aril and the whole fruit were found to be correlated with the pericarp weight, irrespective of the fruit having normal or aborted seed (Huang and Qiu, 1987). In other words, the final fruit size of litchi is determined by the developmental space provided by the pericarp. Cell division in the pericarp of litchi was found to be ceased about 47 days after anthesis (Li et al., 2003b). The present work showed that the period of stage I for 'Siliangguo', 'Chenzi' and EA fruits was about 49 d, while only 39 d for LA fruits. We also found that fruits of large-fruit phenotype (i.e., EA fruits) or genotype (i.e., 'Siliangguo') not only show higher cell division activity, but also exhibit a longer period with active cell division in stage I when compared to those of small-fruit phenotype (i.e., LA fruits) or genotype (i.e., 'Chenzi'). These higher activity and longer period of cell division could likely contribute to the large fruit size observed in EA fruits and 'Siliangguo', which is consistent with the results in melon, where the large-fruit genotype Fuyu A maintains a longer cell division period than the small-fruit genotype Natsu 4 (Kobayashi et al., 2002). Moreover, the transgenic large melon fruits possess higher cell division activity than the control (Kobayashi et al., 2003). In avocado phenotypically small cv Hass fruit can be induced by reduced cell number, which mainly resulted from early cell division (Cowan et al., 1997). Hence, high cell division activity in early stage of fruit development is a prerequisite for a large fruit.

HMGRs have been implicated to play essential roles in cell division during fruit early developmental stage, therefore influencing the final fruit size (Cowan et al., 1997; Enjuto et al., 1994; Kato-Emori et al., 2001; Narita and Gruissem, 1989). In this study, *LcHMG1* was found to actively express in early young fruit and other young tissues which are undergoing rapid cell division, consistent with earlier studies in tomato and melon that both high level of HMGR activity and mRNA expression were detected in the early stages of fruit development (Kobayashi et al., 2002; Narita and Gruissem, 1989). HMGRs have also been investigated in certain woody fruit crops, including

apple from which two HMGR genes have been cloned (Rupasinghe et al., 2001), but their relationship with cell division as well as fruit size remains to be characterized, and avocado where the HMGR enzyme activity was demonstrated to be highly associated with early cell division and to play central role in fruit size regulation (Cowan, 2004; Cowan et al., 1997, 2001), but there is still no report of HMGR gene isolation in this plant. To the best of our knowledge, the present work is the first study in a woody fruit crop to reveal that HMGR gene (*LcHMG1*) expression level is highly associated with cell division during the early stage of fruit development, a factor which determines the final fruit size, suggesting that *LcHMG1* is likely to play an essential role in regulation of litchi fruit size by modulating early cell division.

4.3. LcHMG2 is mainly involved in regulation of the isoprenoid biosynthesis required for cell enlargement

In plants expression of HMGR genes appears to be modulated in response to specific developmental signals. Two peaks of HMGR mRNA accumulation during tomato fruit development were observed (Gillaspy et al., 1993; Narita and Gruissem, 1989). The first peak occurs during early fruit development and the second during ripening. These peaks are due to differential transcription of two HMGR genes. *HMG1* is responsible for the early peak correlated with high cell division activity, and *HMG2* for the ripening-associated peak (Daraselia et al., 1996; Gillaspy et al., 1993), suggesting that tomato HMG1 and HMG2 expression is correlated with cell division and enlargement during fruit development, respectively. Melon that contains a single HGMR gene also follows a similar two-peak expression pattern of HMGR gene during fruit development (Kato-Emori et al., 2001). This two-peak expression pattern of HMGR genes was verified to some extent by our results of expression analysis. *LcHMG1* expression, as described above, was highly correlated with the early rapid cell division while *LcHMG2* was active in the aril during the late stage (ripening) when the rapid enlargement of aril cell is undergoing. Thus like the tomato *HMG2* gene, the litchi *LcHMG2* gene may be more actively expressed during ripening probably to supply enough isoprenoid compounds required for late cell enlargement.

4.4. LcHMG1 probably plays a more essential role in litchi fruit size determination

HMGR enzyme is an essential control point for the isoprenoid metabolism in plants (Chappell et al., 1995). The active *LcHMG1* expression during early stages is likely to satisfy the increasing demand of sterols for the production of cell membrane. Inhibition of HMGR activity causes the appearance of symptoms usually associated with the small-fruit phenotype (Cowan et al., 1997) and reduces endogenous sterol content (Bach, 1995). Transgenic plants with the overexpression of HMGR genes overproduce sterols (Hey et al., 2006; Muñoz-Bertomeu et al., 2007; Schaller et al., 1995). However, the addition of stigmasterol (a major type of sterols in plants) to avocado fruit during early phase reduced fruit growth instead of stimulating it as expected (Cowan et al., 1997), indicating that some other factors must be involved in this regulation. In small avocado fruit reduced HMGR activity was associated with an increased mesocarp ABA concentration, and an imbalance in the ratio of CK/ABA would be expected to reduce cell division and fruit size (Moore-Gordon et al., 1998). In litchi, fruits of large-fruit pheno- or genotype obtained a higher CK/ABA ratio than those of small-fruit pheno- or genotype correspondingly during early stage of fruit development (Li et al., 2005). Thus, it is plausible that *LcHMG1* plays an efficient role in maintaining the sterol biosynthesis and the hormone homeostasis which are vital for cell division during early fruit.

In general, the comparable expression pattern between *LcHMG1* and tomato *HMG1* revealed their greater resemblance, and that *LcHMG1*, instead of *LcHMG2*, is required for early litchi fruit growth, which is the critical stage for final fruit determination. The longer and higher expression of *LcHMG1* is significant to help us explain the size difference between fruits of different genotypes and phenotypes. *LcHMG2* is more likely to be involved in the ripening phrase, which is less related to fruit size determination in litchi. Thus, as far as their roles of two litchi HMGR genes involved in regulating final fruit size were concerned, *LcHMG1* probably plays a more essential role than *LcHMG2* in regulating the formation of litchi fruit size. Further functional characterization of *LcHMG1* will have far-reaching impacts on uncovering the fruit size determination mechanism.

5. Conclusion

HMGRs play an essential role in fruit size determination through the regulation of early cell division. Two HMGR genes (*LcHMG1* and *LcHMG2*) were isolated from fruits of a subtropical fruit crop litchi, and their expression profiles were compared between fruits of different phenotypes or genotypes. We found that two HMGR genes serve distinct roles during the development of litchi fruit. The substantially high expression of *LcHMG1* was correlated with the high level of cell division in the early stage of fruit development, which is the key factor affecting the final fruit size. The fact that LcHMG1 achieved higher levels of expression for longer duration in large-fruited types than in small-fruited types will be helpful to explain the fruit unevenness in different pheno- or genotypes. In contrast, *LcHMG2* was most highly expressed in the late stage of fruit development, in association with biosynthesis of isoprenoid compounds required for later cell enlargement.

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References

Aoyagi, K., Beyou, A., Moon, K., Fang, L., Ulrich, T., 1993. Isolation and characterization of cDNAs encoding wheat 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiol. 102, 623–628.
Bach, T.J., 1995. Some new aspects of isoprenoid biosynthesis in plants. Lipids 30, 191–202.
Bohner, J., Bangerth, F., 1988. Cell number, cell size and hormone levels in semi-isogenic mutants of *Lycopersicon pimpinellifolium* differing in fruit size. Physiol. Plant. 72, 316–320.
Caelles, C., Ferrer, A., Balcells, L., Hegardt, F.G., Boronat, A., 1989. Isolation and structural characterization of a cDNA encoding *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Mol. Biol. 13, 627–638.
Campos, N., Boronat, A., 1995. Targeting and topology in the membrane of plant 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Cell 7, 2163–2174.
Chappell, J., 2002. The genetics and molecular genetics of terpene and sterol origami. Curr. Opin. Plant Biol. 5, 151–157.
Chappell, J., Wolf, F., Proulx, J., Cuellar, R., Saunders, C., 1995. Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants? Plant Physiol. 109, 1337–1343.
Cheng, G.W., Breen, P.J., 1992. Cell count and size in relation to fruit size among strawberry cultivars. J. Am. Soc. Hortic. Sci. 117, 946–950.
Choi, D., Ward, B.L., M., B.R., 1992. Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. Plant Cell 4, 1333–1344.
Chye, M.L., Tan, C.T., Chua, N.H., 1992. Three genes encode 3-hydroxy-3-methylglutaryl-coenzyme-A reductase in *Hevea brasiliensis*: *HMG1* and *HMG3* are differentially expressed. Plant Mol. Biol. 19, 473–484.
Cong, Y., Li, H., Yan, Z.M., Yu, M.L., Chang, Y.H., 2009. The relationship between expression patterns of two members of HMGR gene family and superficial scald in early maturity sandy pear 'Cuiguan' during shelf life. Acta Hortic. Sin. 36, 959–966.
Coombe, B.G., 1973. The regulation of set and development of the grape berry. Acta Hortic. 34, 261–274.

Cowan, A.K., 2004. Metabolic control of avocado fruit growth: 3-hydroxy-3-methylglutaryl coenzyme a reductase, active oxygen species and the role of 7C sugars. (Special issue: Biotechnology, the African perspective.). S. Afr. J. Bot. 70, 75–82.
Cowan, A.K., Moore-Gordon, C.S., Bertling, I., Wolstenholme, B.N., 1997. Metabolic control of avocado fruit growth. Isoprenoid growth regulators and the reaction catalysed by 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiol. 114, 511–518.
Cowan, A.K., Cripps, R.F., Richings, E.W., Taylor, N.J., 2001. Fruit size: towards an understanding of the metabolic control of fruit growth using avocado as a model system. Physiol. Plant. 111, 127–136.
Cruz-Castillo, J.G., Woolley, D.J., Lawes, G.S., 2002. Kiwifruit size and CPPU response are influenced by the time of anthesis. Sci. Hortic. 95, 23–30.
Daraselia, N.D., Tarchevskaya, S., Narita, J.O., 1996. The promoter for tomato 3-hydroxy-3-methylglutaryl coenzyme A reductase gene 2 has unusual regulatory elements that direct high-level expression. Plant Physiol. 112, 727–733.
Eisenreich, W., Rohdich, F., Bacher, A., 2001. Deoxyxylulose phosphate pathway to terpenoids. Trends Plant Sci. 6, 78–84.
Enjuto, M., Balcells, L., Campos, N., Caelles, C., Arró, M., Boronat, A., 1994. *Arabidopsis thaliana* contains two differentially expressed 3-hydroxy-3-methylglutaryl-CoA reductase genes, which encode microsomal forms of the enzyme. Proc. Natl. Acad. Sci. U. S. A. 91, 927–931.
Gillaspy, G., Ben-David, H., Gruissem, W., 1993. Fruits: a developmental perspective. Plant Cell 5, 1439–1451.
Goldstein, J.L., Brown, M.S., 1990. Regulation of the mevalonate pathway. Nature 343, 425–430.
Hey, S.J., Powers, S.J., Beale, M.H., Hawkins, N.D., Ward, J.L., Halford, N.G., 2006. Enhanced seed phytosterol accumulation through expression of a modified HMG-CoA reductase. Plant Biotechnol. J. 4, 219–229.
Huang, H.B., Qiu, Y.X., 1987. Growth correlation and assimilate partitioning in the arillate fruit of *Litchi chinensis* Sonn. Aust. J. Plant Physiol. 14, 181–188.
Huang, H.B., Xu, J.K., 1983. The developmental patterns of fruit tissues and their correlative relationships in *Litchi chinensis* Sonn. Sci. Hortic. 19, 335–342.
Istvan, E.S., Palnitkar, M., Buchanan, S.K., Deisenhofer, J., 2000. Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. EMBO J. 19, 819–830.
Kato-Emori, S., Higashi, K., Hosoya, K., Kobayashi, T., Ezura, H., 2001. Cloning and characterization of the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase in melon (*Cucumis melo* L. reticulatus). Mol. Genet. Genomics 265, 135–142.
Kevei, Z., et al., 2007. 3-hydroxy-3-methylglutaryl coenzyme A reductase1 interacts with NORK and is crucial for nodulation in *Medicago truncatula*. Plant Cell 19, 3974–3989.
Kobayashi, T., Kato-Emori, S., Tomita, K., Ezura, H., 2002. Detection of 3-hydroxy-3-methylglutaryl-coenzyme A reductase protein Cm-HMGR during fruit development in melon (*Cucumis melo* L.). Theor. Appl. Genet. 104, 779–785.
Kobayashi, T., Kato-emori, S., Tomita, K., Ezura, H., 2003. Transformation of tomato with the melon 3-hydroxy-3-methylglutaryl-coenzyme A reductase leads to increase of fruit size. Plant Biotechnol. 2003, 297–303.
Lai, R., Woolley, D.J., Lawes, G.S., 1990. The effect of inter-fruit competition, type of fruiting lateral and time of anthesis on the fruit-growth of kiwifruit (*Actinidia deliciosa*). J. Hortic. Sci. 65, 87–96.
Lange, B.M., Rujan, T., Martin, W., Croteau, R., 2000. Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc. Natl. Acad. Sci. U. S. A. 97, 13172–13177.
Li, J., Huang, X., Huang, H., Zhou, B., 2002. A cytological and physiological study of large- and small-sized litchi cultivars fruit. J. Fruit Sci. 19, 158–162.
Li, J.G., Huang, H.B., Huang, X.M., 2003a. A revised division of the developmental stages in litchi fruit. Acta Hortic. Sin. 33, 307–310.
Li, J.G., Huang, H.B., Liu, X.D., 2003b. Cytological studies of litchi pericarp during development. Acta Hortic. Sin. 30, 23–28.
Li, J., Huang, H., Huang, X., 2004. Differential fruit size of 'Feizixiao' litchi fruit from early and late bloom as affected by temperature regimes. J. Fruit Sci. 21, 37–41.
Li, J.G., Zhou, B.Y., Huang, X.M., Huang, H.B., 2005. The roles of cytokinins and abscisic acid in the pericarp of litchi (*Litchi chinensis* Sonn.) in determining fruit size. J. Hortic. Sci. Biotechnol. 80, 571–574.
Li, J.G., Huang, X.M., Huang, H.B., 2010. An overview of factors related to fruit size in *Litchi Chinensis* Sonn. Acta Hortic. 863, 477–482.
Li, C.Q., Wang, Z.H., Xu, Y.S., Zhang, J.A., Li, J.G., 2011. Improvement of the cell nuclei suspensions for flow cytometry and comparison on the cell division activity of litchi fruitlet and pericarp which having different final fruit size. Acta Hortic. Sin. 38, 1781–1790.
Liao, Z.H., et al., 2004. Cloning and characterisation of the gene encoding HMG-CoA reductase from *Taxus media* and its functional identification in yeast. Funct. Plant Biol. 31, 73–81.
Loguercio, L.L., Scott, H.C., Trolinder, N.L., Wilkins, T.A., 1999. Hmg-coA reductase gene family in cotton (*Gossypium hirsutum* L.): unique structural features and differential expression of hmg2 potentially associated with synthesis of specific isoprenoids in developing embryos. Plant Cell Physiol. 40, 750–761.
Lu, W.J., Jiang, Y.M., 2003. Cloning and sequence analysis of expansin genes in Litchi chinensis Sonn. fruits. Sci. Agric. Sinica 36, 1525–1529.
Marguery, P., Sangwan, B.S., 1993. Sources of variation between apple fruits within a season, and between seasons. J. Hortic. Sci. 68, 309–315.
Monfar, M., Caelles, C., Balcells, L., Ferrer, A., Hegardt, F.G., Boronat, A., 1990. Molecular cloning and characterization of plant 3-hydroxy-3-methylglutaryl coenzyme A reductase. In: G.H.N., T., H.A., S. (Secondary Authors) (Eds.), Biochemistry of the Mevalonic Acid Pathway to terpenoids, Recent Advances in Phytochemistry. Plenum press, New York and London, pp. 83–97.
Moore-Gordon, C.S., Cowan, A.K., Bertling, I., Botha, C.E.J., Cross, R.H.M., 1998. Symplastic solute transport and avocado fruit development: a decline in cytokinin/ABA ratio is related to appearance of the Hass small fruit variant. Plant Cell Physiol. 39, 1027–1038.
Muñoz-Bertomeu, J., Sales, E., Ros, R., Arrillaga, I., Segura, J., 2007. Up-regulation of an N-terminal truncated 3-hydroxy-3-methylglutaryl CoA reductase enhances production of essential oils and sterols in transgenic *Lavandula latifolia*. Plant Biotechnol. J. 5, 746–758.
Narita, J.O., Gruissem, W., 1989. Tomato hydroxymethylglutaryl-CoA reductase is required early in fruit development but not during ripening. Plant Cell 1, 181–190.
Nelson, A.J., Doerner, P.W., Zhu, Q., Lamb, C.J., 1994. Isolation of a monocot 3-hydroxy-3-methylglutaryl coenzyme A reductase gene that is elicitor-inducible. Plant Mol. Biol. 25, 401–412.
Otto, F., 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Zbigniew, D., Harry, A.C. (Eds.), Methods in Cell Biology. Academic Press, pp. 105–110.
Praloran, J.C., Vullin, G., Jaquemond, C., Depierre, D., 1981. Observations on the growth of clementines in Corsica. Fruits 36, 755–767.
Rodriguez-Concepcion, M., Boronat, A., 2002. Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and blastids. A metabolic milestone achieved through genomics. Plant Physiol. 130, 1079–1089.
Rodriguez-Concepcion, M., Gruissem, W., 1999. Arachidonic acid alters tomato *HMG* expression and fruit growth and induces 3-hydroxy-3-methylglutaryl coenzyme A reductase-independent lycopene accumulation. Plant Physiol. 119, 41–48.
Rodriguez-Concepcion, M., et al., 2004. Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during *Arabidopsis* seedling development. Plant Cell 16, 144–156.
Rohmer, M., Seemann, M., Horbach, S., BringerMeyer, S., Sahm, H., 1996. Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. J. Am. Chem. Soc. 118, 2564–2566.
Rupasinghe, H.P.V., Almquist, K.C., Paliyath, G., Murr, D.P., 2001. Cloning of *hmg1* and *hmg2* cDNAs encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase and their expression and activity in relation to alpha-farnesene synthesis in apple. Plant Physiol. Biochem. 39, 933–947.
Sando, T., et al., 2008. Cloning and characterization of mevalonate pathway genes in a natural rubber producing plant, *Hevea brasiliensis*. Biosci. Biotechnol. Biochem. 72, 2049–2060.
Schaller, H., et al., 1995. Expression of the *Hevea brasiliensis* (H.B.K.) Mull. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. Plant Physiol. 109, 761–770.
Scorza, R., May, L.G., Purnell, B., Upchurch, B., 1991. Differences in number and area of mesocarp cells between small-fruited and large-fruited peach cultivars. J. Am. Soc. Hortic. Sci. 116, 861–864.
Stermer, B.A., Bianchini, G.M., Korth, K.L., 1994. Regulation of HMG-CoA reductase activity in plants. J. Lipid Res. 35, 1133–1140.
Wan, C.Y., Wilkins, T.A., 1994. A modified hot borate method significantly enhances the yield of high quality RNA from cotton (Gossypium hirsutum L.). Anal. Biochem. 223, 7–12.
Xia, R., Lu, W.J., Li, J.G., 2008. A modified method of amplifying the 5-end cDNA sequence. Acta Hortic. Sin. 35, 1533–1538.
Xia, R., Lu, W.J., Wang, Z.H., Li, J.G., 2011. Cloning and characterization of two genes for 3-hydroxy-3-methylglutaryl coenzyme A reductase and their possible roles during fruit development in *Dimocarpus longan*. J. Hortic. Sci. Biotechnol. 86, 25–30.