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Cloning and characterisation of two genes for 3-hydroxy-3-methylglutaryl coenzyme A reductase and their possible roles during fruit development in *Dimocarpus longan*

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SUMMARY
3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC: 1.1.1.34), encoded by multiple genes in eukaryotes, is known to catalyse the first committed step in the mevalonic acid (MVA) pathway for the biosynthesis of isoprenoids, and has been implicated in fruit size determination. To elucidate whether the genes for HMGR are involved in the regulation of fruit growth in longan (*Dimocarpus longan*), we cloned two sequence-distinct HMGR genes, designated *DlHMG1* and *DlHMG2*, respectively. Sequence analyses revealed that they shared a high level of sequence and domain conservation, with homologues identified in other species at both the nucleotide and amino acid levels. Gene expression analysis showed that the two genes were differentially regulated in the pericarp, arils, and seed, as well as during fruit development. *DlHMG1* was up-regulated particularly in Phase I [0 – 42 d after anthesis (DAA)] and at the early stage of Phase II (42 – 56 DAA), followed by down-regulation in Phase III (≥ 56 DAA); while *DlHMG2* was expressed constitutively in the pericarp and arils, but fluctuated periodically in seed tissues during fruit development. The possible roles of *DlHMG1* and *DlHMG2* in the regulation of longan fruit size and growth are discussed.

Longan (*Dimocarpus longan*), with its unique flavour and high nutritional value, is recognised as one of the most valuable commercial fruits cultivated in southern China. However, lack of a uniform fruit size in most varieties compromises its quality and commercial value. It has been reported that the key factor that determines fruit size is cell number, rather than cell volume, in most fruits (Cowan *et al*., 2001; Olmstead *et al*., 2007). Any factors that affect cell division activity and processes should therefore play a role in the determination of fruit size and shape. Recently, genes coding for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC: 1.1.1.34) were reported to be involved in fruit development through the regulation of cell division (Cowan *et al*., 2001). HMGR genes that belong to a gene family have been characterised extensively in numerous species, including *Arabidopsis thaliana* (Caelles *et al*., 1989; Enjuto *et al*., 1994), tomato (Narita and Grussem, 1989), rice (Nelson *et al*., 1994), corn (Stermer *et al*., 1994), wheat (Aoyagi *et al*., 1993), *Salvia miltiorrhiza* (Liao *et al*., 2009), apple (Rupasinghe *et al*, 2001), *Camptotheca acuminate* (Maldonado-Mendoza *et al*., 1997), rubber (Chye *et al*., 1992), and *Taxus media* (Liao *et al*., 2004). HMGR activity has been found to be associated with cell division in many fruits, including tomato (Narita and Grussem, 1989), avocado (Cowan *et al*., 1997), melon (Kato-Emori *et al*., 2001), and apple (Rupasinghe *et al*., 2001). Direct evidence for HMGR being an important regulator of fruit size was obtained through transgenic plant research. Over-expression of a melon *HGMR* gene in tomato greatly stimulated cell division activity, leading to the production of much larger fruit in transgenic plants compared to fruit in wild-type tomato plants (Kobayashi *et al*., 2003).

HMGR activity and function are highly conserved in eukaryotes, and the biochemical properties of the enzyme have been fully elucidated. HMGR catalyses the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate (MVA; Caelles *et al*., 1989; Goldstein *et al*., 1990), the first committed step in cytosolic isoprenoid biosynthesis (Goldstein *et al*., 1990; Campos and Boronat, 1995). The reaction is irreversible and is considered to be the rate-limiting step in the biosynthesis of isopentenyl pyrophosphate (IPP) (Chappell, 1995; Rupasinghe *et al*., 2001). Isoprenoids are widespread in eukaryotes and are a large and diverse group of compounds with different structures (Langer *et al*., 2000). They are derived from the common precursor, IPP, and represent many major vital compounds that are involved in a wide range of primary and secondary pathways of metabolism in plants. For example, three of the five major groups of growth regulators (i.e., abscisic acid, gibberellins, and cytokinins), as well as chlorophylls and plastoquinone in photosynthesis, carotenoids in photo-protection, and some phytoalexins and steroids required for the assembly of biological membranes, are isoprenoids that are essential for plant growth, development, and defence (Stermer *et al*., 1994; Cowan *et al*., 1997).

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In this study, we have isolated and characterised two HMGR genes (*DlHMG1* and *DlHMG2*) from longan (*Dimocarpus longan*), in order to understand the roles of these genes in the regulation of longan fruit development and size determination, which are major concerns for longan fruit production. We found that *DlHMG1* was specifically up-regulated in the early stages of fruit development, while *DlHMG2* was not, implying that *DlHMG1* may be a key regulator of fruit growth, as has been demonstrated in other species.

MATERIALS AND METHODS

Materials

Three 7-year-old 'Linglong' longan plants (*Dimocarpus longan*), grown in the orchard of South China Agricultural University, were used for tissue collection. Fruit collection started 14 d after anthesis (DAA), followed by weekly collections until the fruit were ripe. Ten fruit, located on different panicles on each tree, were labelled for monitoring fruit stage and size development. Ten-to-twenty fruit, of sizes similar to the labelled fruit, were collected from each tree and immediately stored in an icebox before dissection into pericarp, aril, and seed tissues, each of which were weighed and stored at –80ºC in a freezer.

Total RNA extraction and preparation of cDNA

Total RNA was isolated from approx. 10 g of each type of frozen fruit tissue using the hot borate method (Wan and Wilkins, 1994; Lu and Jiang, 2003). RNA was reverse-transcribed using 10 Units Reverse Transcriptase (XL AMV; TaKaRa, Dalian, P. R. China) and oligo(dT) primer. Degenerate primers were designed for cloning the longan HMGR genes according to the methods and principles described by Xia *et al*. (2006). PCR for amplification of a *DlHMG1* DNA fragment was conducted using total cDNA as template with the forward primer (5'-GGTTACGAGTATTGGGTTCCT atggcnacnac-3'; GREYSVPMATT) and the reverse primer (5'-GAACTCTCAACATTTTGTGCAggttcyt gncc-3'; for the corresponding amino acid sequence GQDPAQNVESS). For *DlHMG2*, PCR amplification used the forward primer (5'-CTTCTTTTATTTATC TGCTGGGCttytkyggnatc-3'; SFIYLLGFFGI) and the reverse primer (5'-CCCTTAGAAACCATATTCATA cccatngcrtc-3'; DAMGMNMVSKG). Both PCR amplifications were performed in total volumes of 50 µl containing 5 µl 10 × *Ex* PCR buffer, 4 µl dNTP mixture, 2 µl forward primer (10 µM), 2 µl reverse primer (10 µM), 2 µl cDNA, and 5 Units *Ex Taq* DNA polymerase (TaKaRa). The same protocol, consisting of 35 amplification cycles [94ºC for 1 min, the T_m (annealing temperature) for 1 min, and 72ºC for 1 min] was used to amplify both genes, except that the T_m was 58.5ºC for *DlHMG1* and 56ºC for *DlHMG2*. Two fragments of the expected sizes (700 bp for *DlHMG1* and 800 bp for *DlHMG2*) were obtained and cloned into the vector, pMD 18-T (TaKaRa). Both fragment 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/) using BLASTX to confirm the sequences.

Gene-specific primers for each gene fragment (5'-AGGTGGTGAAGAAGGTGTTG-3' and 5'-CTTACT GGCTCTGCTGTTGC-3' for *DlHMG1*; and 5'-TTTTC CTGGAGAATCCTGCC-3' and 5'-TTGGAAGGC TTCAGAACATC-3' for *DlHMG2*) were then designed and used for 3'-RACE analysis (3'-Full RACE Core Set Version 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 (5'-GACGGACGACAA GAC-3' for *DlHMG1*; or 5'-TCGTCCTCGCTGGTA-3' for *DlHMG2*). The nested-PCR strategy was used for amplifications with the gene-specific primers (5'-TTTCGGCAACGGAGTAGGCG-3' and 5'-CGTGAG ATGAAGGACTGGAC-3' for *DlHMG1*; and 5'-CTTA GCCGTGACGAGAGAGC-3' and 5'-CCACATTTCG GAGGAGGGAC-3' for *DlHMG2*). The amplified bands were cloned and sequenced, as described above.

Sequence analysis

Full-length cDNA sequences for *DlHMG1* and *DlHMG2* were assembled through sequential ligation and cloning, and verified by DNA sequencing. Multiple sequence alignment analysis was performed using ClustalW (http://www.ebi.ac.uk/clustalw/). A phylogenetic tree was then constructed by the neighbour-joining method based on the Phylip programme (http://bioweb.pasteur.fr/phylogeny/intro-en.html)

Northern blot analysis

DIG-labelled gene-specific probes were generated using a PCR-based DIG labelling kit (Roche Diagnostics, Mannheim, Germany) with the primers (5'-AGAGACATGACCGAAGCCGC-3' and 5'-GCTCTC AGGTCGTGACAAGG-3' for *DlHMG1*; 5'-ATTGAG GTTGGCACAGTTGG-3' and 5'-CCAGGACTGAAG GGGCTATT-3' for *DlHMG2*) designed following the manufacturer's instructions. Total RNA (10 µg) from each sample was separated by electrophoresis in a formaldehyde-treated 1.2% (w/v) agarose gel, blotted onto a PVDF membrane (Biodyne B; 0.45 µm; PALL, New York, USA), and immediately fixed to the membrane by baking at 80ºC for 2 h, followed by UV cross-linking (Amersham Life Sciences, Amersham, UK). Pre-hybridisation of the membrane was carried out in high-SDS buffer [7% (w/v) SDS, 5× SSC, 50 mM sodium phosphate (pH 7.0), 2% (w/v) blocking reagent (Roche Diagnostics), and 0.1% (w/v) N-lauroylsarcosine, in 50% (v/v) deionised formamide] for at least 3 h, followed by hybridisation with each DIG-labelled probe at 45ºC for 16 h in the same buffer. The blots were washed twice in 2× SSC containing 0.1% (w/v) SDS at 37ºC for 10 min, and twice with 0.1× SSC including 0.1% (w/v) SDS at 62ºC for 30 min before hybrid signal detection by chemiluminescence using CDP-Star (Roche Diagnostics).

RESULTS AND DISCUSSION

Isolation and characterisation of the DlHMG1 *and* DlHMG2 *genes*

In all plant species analysed so far, HGMR is encoded by a multigene family (Campos and Boronat,

1995), but the number of genes in the family varies from species-to-species (Chappell, 1995). Using PCR amplification, in combination with 5'- and 3'-RACE analyses, we isolated two sequence-distinct HMGR genes in longan, called *DlHMG1* and *DlHMG2*, respectively. *DlHMG1* cDNA (2,235 bp in length), consisted of a 56 bp 5'-untranslated region (5'-UTR), a 1,695 bp open reading frame (ORF), and a 484 bp 3' untranslated region (3'-UTR; Figure 1A). *DlHMG1* encoded a putative polypeptide of 564 amino acids. Similarly, *DlHMG2* cDNA (Figure 1B) had a 133 bp 5'-UTR, a 1,707 bp ORF, and a 359 bp 3'-UTR. The *DlHMG2* ORF encoded a putative polypeptide of 568 amino acids, which was four amino acids longer than DlHMG1.

A BLASTX search against GenBank showed that DlHMG1 shared ≥ 75% sequence identity with other HMGR proteins in many species, but the highest level of identity (94%) was with litchi (*Litchi chinensis*) HMGR (ABF56518). DlHMG2 appeared to be less conserved in plants than DlHMG1, and the highest level of identity was with the *Gossypium hirsutum* HMGR (O64967) at approx. 74%. Sequence alignments between the DlHMG1 and DlHMG2 proteins revealed approx. 73% sequence identity, indicating substantial sequence divergence during evolution. Thus, our results indicate that longan plants contain at least two HMGR genes, or homologues, in their genome.

Sequence alignment and phylogenetic analysis

Multiple sequence alignments between DlHMG1 and DlHMG2 and ten other plant HMGR proteins were performed using the ClustalW programme (Figure 2). Similar to other plant HMGRs, DlHMG1 and DlHMG2 contained four conserved regions (domains), defined as the N-terminal region, a membrane domain, a linker region, and a catalytic domain (Figure 2A; Monfar *et al*., 1990; Campos and Boronat, 1995; Rupasinghe *et al*., 2001). Among these, the membrane and the catalytic domains were highly conserved, whereas the N-terminal and the linker regions were highly divergent, both in length and in amino acid sequence (Campos and Boronat, 1995). The existence of two highly-conserved hydrophobic sequences (H1 and H2) in the membrane domain suggested that both these HMGRs were targeted to the endoplasmic reticulum (ER; Caelles *et al*., 1989; Campos and Boronat, 1995). Two HMG-CoA binding motifs [EMPV(I)GYVQV(I)P and TTEGCLVA] and two NADPH-binding motifs (DAMGMNM and GTVGGGT) were highly conserved in the catalytic domain (Figure 2B; Wang *et al*.., 1990; Liao *et al*.., 2004; Istvan and Deisenhofer, 2000). Most plant HMGRs contain a MetAsp/GluXArgArgArg motif [MD/EXRRR (where X can be Val, Leu, Ile, or Pro)] at their N-terminus, which might anchor the proteins in the ER (Campos and Boronat, 1995). These results strongly suggest that DlHMG1 and DlHMG2 could be

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1                                                    ttttcaatttgttatttttagtcctctcaaaatcgagtttcggcgaaagtcgaata
57  atggacgtccgccgacggcctccgaagccgtcacgtgtcgccgacgatgaacagcgtactacgacgccgtctccgcagtcgccgaaagca
      M  D  V  R  R  R  P  P  K  P  S  R  V  A  D  D  E  Q  R  T  T  T  P  S  P  Q  S  P  K  A
147 tcagatgctttgccgctgccactatacctaacgaacgccatttcttcacgctcttcttctcggtggcgtactttcctgctacacaggtgg
      S  D  A  L  P  L  P  L  Y  L  T  N  A  I  F  F  T  L  F  F  S  V  A  Y  F  L  L  H  R  W
237 cgcgacaagatccgtagctcgacgcctctccacgtcgtcactctctctgaaatcgccgccattgtctccctcatcgcctctttcatctac
      R  D  K  I  R  S  S  T  P  L  H  V  V  T  L  S  E  I  A  A  I  V  S  L  I  A  S  F  I  Y
327 cttctcggcttcttcggcatagactttgtccagtccttcatctcacgcgcctccaatgacgcgtgggatctagacgacgaggctgcaacc
      L  L  G  F  F  G  I  D  F  V  Q  S  F  I  S  R  A  S  N  D  A  W  D  L  D  D  E  A  A  T
417 gtcattgccgctcgtcctccgcctactccgttgccgaaactggtcgctcctcccgagccgataatatctgtcttgtcgtccgtcgaagat
      V  I  A  A  R  P  P  P  T  P  L  P  K  L  V  A  P  P  E  P  I  I  S  V  L  S  S  V  E  D
507 gagaagatcgtgaagtcggtcgtggacggctcgattccgtcgcactcgctggagtcgaagctcggggactgtaaaagagcggctctgatt
      E  K  I  V  K  S  V  V  D  G  S  I  P  S  H  S  L  E  S  K  L  G  D  C  K  R  A  A  L  I
597 cgacggaaggcgctgcagacagtgactggtagatcactgcaggggctgccgttggatggcttcgattatgagtcgatccaggggcagtgc
      R  R  K  A  L  Q  T  V  T  G  R  S  L  Q  G  L  P  L  D  G  F  D  Y  E  S  I  Q  G  Q  C
687 tgcgaaatgccggttggttacgtgcagattccggtgggaatcgccgggcctctgcttttagacgggttcgagtattcggttccaatggca
      C  E  M  P  V  G  Y  V  Q  I  P  V  G  I  A  G  P  L  L  L  D  G  F  E  Y  S  V  P  M  A
777 acgacggagggctgtttggttgccagcgcaaacagaggctgcagggcgatctacgcctccggtggcgcctccactgtgctgttgaaagac
      T  T  E  G  C  L  V  A  S  A  N  R  G  C  R  A  I  Y  A  S  G  G  A  S  T  V  L  L  K  D
867 ggtatgaccagagctcctgttgtgaggtttccaccgccaagagagctgccgagttgaagttcttcttggaagatcctaacaatttcgat
      G  M  T  R  A  P  V  V  R  F  S  T  A  K  R  A  A  E  L  K  F  F  L  E  D  P  N  N  F  D
957 acattagctgtggttttaacaggtcgagtaggcttgccaggcttcgaagcattcaatgctctattgctggaaagaatctctacactagg
      T  L  A  V  V  F  N  R  S  S  R  L  A  R  L  R  S  I  Q  C  S  I  A  G  K  N  L  Y  T  R
1047 tttgttgtagcacgggtgacgccatggggatgaacatggtttccaaaggagttcaaaatgttcttgatttccttcaaaacgattatcct
      F  C  C  S  T  G  D  A  M  G  M  N  M  V  S  K  G  V  Q  N  V  L  D  F  L  Q  N  D  Y  P
1137 gacatggatgttattggaaatatctggaaacttctgttcggacaaaaaacctgctgctgtaaattggattgaagggcgtggtaaatcagtt
      D  M  D  V  I  G  I  S  G  N  F  C  S  D  K  K  P  A  A  V  N  W  I  E  G  R  G  K  S  V
1227 gtttgcgaggctataatcaaggaagaggtggtgaagaaggtgttgaaaacaaacgtagcttcattagttgagcttaatatgctcaagaac
      V  C  E  A  I  I  K  E  E  V  V  K  K  V  L  K  T  N  V  A  S  L  V  E  L  N  M  L  K  N
1317 cttactggctctgctgttgccggtgccctgggtggatttaatgcccatgctgccaatattgtctctgcaatctacttagccactggtcag
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1407 gatccggcacaaaatgttgagagttctcattgcattaccatgatggaagctgtcaatgacgggagggatttgcatgtttctgtaactatg
      D  P  A  Q  N  V  E  S  S  H  C  I  T  M  M  E  A  V  N  D  G  R  D  L  H  V  S  V  T  M
1497 ccatccattgaggtgggtacagttggtggtggtactcaactcgcatctcaatctgcttgcctgaatttgctgggtgtgaagggtgcaagc
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1587 aaagagtcaccaggatcaaatgctaggctcttggccactattgtagctggttcagtcctggcaggggagctctccgtgatgtctgccatt
      K  E  S  P  G  S  N  A  R  L  L  A  T  I  V  A  G  S  V  L  A  G  E  L  S  V  M  S  A  I
1677 gcagctggtcatcttgtcagaagtcacatgaaatacaacagatccagcagagacatgaccgaagccgcatcatagatggagatgtgaact
      A  A  G  H  L  V  R  S  H  M  K  Y  N  R  S  S  S  R  D  M  T  E  A  A  S  *
     agaaatataaaattcaagagaaaggtggacacaatgtaccggagtggaagaagcaaaaatgtaaaatctattggtaacagtgcttgtgta
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     ggctaaaaaaaaaaaaaaa
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B

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1                                                    aaaactcaaccgaattttaattaaatatttataatacaaatcc
44  accactccctgcaattacaacactatttttcattttggcaaccctcgctctctcggatctctccgtcgccggccgccaccgccgtcttca
134 atggagcctcgccggcgatccgcgtccaagcaatctcatctcgatgcgaaggccctcgatggcacgacgaaggcgtcggacgcgctgcct
      M  E  P  R  R  R  S  A  S  K  Q  S  H  L  D  A  K  A  L  D  G  T  T  K  A  S  D  A  L  P
224 ctgccgctctacataacgaacgcggttttcctcacgctcttcttcacggtggtgtactacctgctctcgcgatggcggagaaggtccgg
      L  P  L  Y  I  T  N  A  V  F  L  T  L  F  F  T  V  V  Y  Y  L  L  S  R  W  R  E  K  V  R
314 acgtcgacgccgctccacgtggtgactatgtcggagatgggtgccatcgtggcgttcgtggcgtcctgcatctacctgctcggcttcttc
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404 gggatcgacttcgtgcagtcactcctcttccgtccctcctccgaaatgtggacggaagaagaggatgaggttatcatcaaggaagattcg
      G  I  D  F  V  Q  S  L  L  F  R  P  S  S  E  M  W  T  E  E  E  D  E  V  I  I  K  E  D  S
494 aggcagttgccttgtggacaggccctcgattgctctctcgtcacggctaagcctgtcaaagtcatcgaaacggtgccggtttccttttacc
      R  Q  L  P  C  G  Q  A  L  D  C  S  L  V  T  A  K  P  V  K  V  I  E  T  V  P  V  S  F  T
584 agcgaggacgacgaggagatagtgaagtcggtggtggaggggacgacgccgtcctactcgttggagtcgaagttgggagattgcaggagg
      S  E  D  D  E  E  I  V  K  S  V  V  E  G  T  T  P  S  Y  S  L  E  S  K  L  G  D  C  R  R
674 gcggcgggcatacggcgggaggcgttgcagaggttgacggggaaatctctggcgggtctgcctttggatggtttcgactacgagtccatt
      A  A  G  I  R  R  E  A  L  Q  R  L  T  G  K  S  L  A  G  L  P  L  D  G  F  D  Y  E  S  I
764 ctggggcagtgctgcgagatgcctgtgggttatgtgcagattccggtggggattgcggggcctttgttgcttgacgggacggagttctca
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854 gttccgatggcgaccacggaagggtgcttggtggccagtactaatagggggttgtaaagccattcacttgtctggtggggcttcaagtgtt
      V  P  M  A  T  T  E  G  C  L  V  A  S  T  N  R  G  C  K  A  I  H  L  S  G  G  A  S  S  V
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1034 gccaatttcgaaactctgtctgtcattttcaacaaatccagtaggtttggaaggcttcagaacatcaaatgtgcgattgctggcaagaat
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1124 ctctacattaggttctgttgcagcacaggtgatgctatggggatgaacatggtctccaagggtgtccagaacgttatggattacctccag
      L  Y  I  R  F  C  C  S  T  G  D  A  M  G  M  N  M  V  S  K  G  V  Q  N  V  M  D  Y  L  Q
1214 gaagagttccctgacatggatgtcattggcatttctggtaacttctgctccgacaagaagcctgcagcagtgaactggattgaagggcgt
      E  E  F  P  D  M  D  V  I  G  I  S  G  N  F  C  S  D  K  K  P  A  A  V  N  W  I  E  G  R
1304 ggcaagtctgtggtttgtgaggctataataaagggtgatgtggtgaggaaggttttgaagactaatgtggaggtcttggttgagctcaac
      G  K  S  V  V  C  E  A  I  I  K  G  D  V  V  R  K  V  L  K  T  N  V  E  V  L  V  E  L  N
1394 atgctcaagaaccttactggctctgttatggccggagctcttggtggcttcaatgctcatgccagtaacattgttactgctgtctactta
      M  L  K  N  L  T  G  S  V  M  A  G  A  L  G  G  F  N  A  H  A  S  N  I  V  T  A  V  Y  L
1484 gccactggtcaagatcctgctcagaatgtcgagagctctcactgc atcactatgatggaggccgttaatgatggcaaggaccttcatgtt
      A  T  G  Q  D  P  A  Q  N  V  E  S  S  H  C  I  T  M  M  E  A  V  N  D  G  K  D  L  H  V
1574 tccgtcactatgccttctattgaggttggcacagttggaggtggcacacagcttccttctcaatcagcttgtttgaatctacttggggtc
      S  V  T  M  P  S  I  E  V  G  T  V  G  G  G  T  Q  L  P  S  Q  S  A  C  L  N  L  L  G  V
1664 aagggtgccagcagagaggtaccaggtgcaaactcaaggcttctggccaccattgtagctggttctgttcttgctggggagctgtcgctc
      K  G  A  S  R  E  V  P  G  A  N  S  R  L  L  A  T  I  V  A  G  S  V  L  A  G  E  L  S  L
1754 ttgtcggctcttgcggctggacagctcgttaggagccacatgaagtataatagatctagcaaagatatgagtaaggtttcttcttaagag
      L  S  A  L  A  A  G  Q  L  V  R  S  H  M  K  Y  N  R  S  S  K  D  M  S  K  V  S  S  *
     aacaatagcccccttcagtcctggacacctcacgaactgttgtaaagatgattcattaaatcatatatatagagagagagagcgcacagg
     attcgtactaggaataaaatggaaaacaaaaaaccgtacctaagcgaagataaacctttcactttatgtactgtttctgtttgtgtcagtt
     ctttctgtttgtaagttttagctctgtaatctacagaaccggtataacgatgaaaagatctagtccattcgctttttcaatatcccctaat
     cgggttgtccagtgctgaaatttaatgtatcatctatttattcatgaatgaatttgatatctaagtttactcaaaaaaaaaaaaaa
```

FIG. 1

Nucleotide sequences and deduced amino acid sequences of *DlHMG1* (Panel A) and *DlHMG2* (Panel B) and their encoded proteins. The amino acid sequences are shown in the one-letter code below the corresponding codons. The underlined nucleotides indicate the untranslated regions. Small asterisks "*" indicate the stop codons, and the boxed codons are the start codons. Bold letters on a grey background show the positions of the conserved motifs in HMGR proteins.

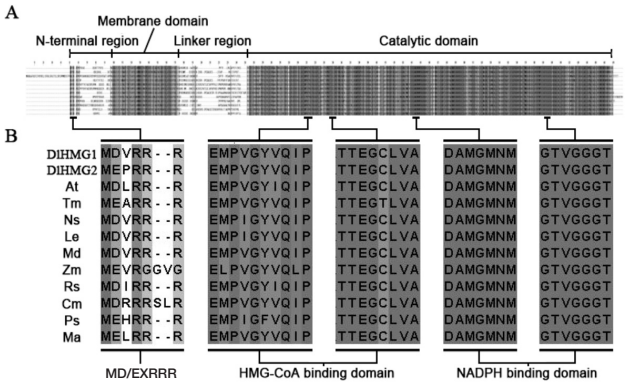

FIG.2
Multiple alignments of DlHMG1 and DlHMG2 with other HGMR proteins from *Arabidopsis thaliana* HMG1 (At; NP_177775), *Cucumis melo* (Cm; BAA36291) *Lycopersicon esculentum* (Le; AAB62581), *Malus* × *domestica* hmg1 (Md; AAK64657), *Morus alba* (Ma; AAD03789), *Nicotiana sylvestris* (Ns; CAA45181), *Pisum sativum* (Ps; AAL37041), *Raphanus sativus* (Rs; CAA48611), *Tilia miqueliana* (Tm; AAY68034), and *Zea mays* (Zm; CAA70440). Panel A, a low-resolution diagram of the full-length alignment of HGMR proteins to show the locations of the conserved motifs only. Panel B, conserved amino acid motifs only.

functionally conserved in their involvement in cytosolic isoprenoid biosynthesis.

A phylogenetic tree was generated from the deduced amino acid sequences of DlHMG1 and DlHMG2, and 21 other homologues (Figure 3). The HMGR proteins clustered into three major groups, those from fungi, vertebrates, and plants. The plant group was also sub-divided into two sub-groups, those from monocotyledons and those from dicotyledons. In the dicotyledon sub-groups, DlHMG1 and DlHMG2 were distributed in two

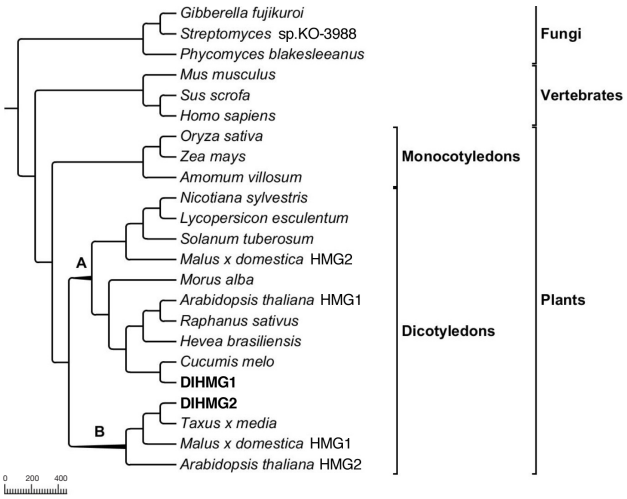

FIG. 3
Phylogenic analysis of DlHMG1 and DlHMG2 (in bold) with other HMGR proteins from *Amomum villosum* (ACR02667), *Arabidopsis thaliana* HMG1 (NP177775), *Arabidopsis thaliana* HMG2 (NP179329), *Cucumis melo* (BAA36291), *Hevea brasiliensis* (AAQ63055), *Homo sapiens* (NP000850), *Gibberella fujikuroi* (CAA63970), *Lycopersicon esculentum* (AAB62581), *Malus* × *domestica* HMG1 (AAK64657), *Malus* × *domestica* HMG2 (ABQ52378), *Morus alba* (AAD03789), *Nicotiana sylvestris* (CAA45181), *Oryza sativa* (AAD08820), *Phycomyces blakesleeanus* (CAB97179), *Raphanus sativus* (CAA48611), *Saccharomyces cerevisiae* (P12683), *Solanum tuberosum* (P48020), *Streptomyces* sp.KO-3988 (BAD86804), *Sus scrofa* (ABF83891), *Taxus media* (AAQ82685), and *Zea mays* (CAA70440). The phylogenetic tree was constructed by the neighbour-joining method based on the Phylip programme (http://bioweb.pasteur.fr/phylogeny/intro-en.html), and bootstrapping was carried out on 500 replicates. The scale represents millions of years of evolution.

distinct classes (A and B). DlHMG1 was located in Class A with HMGR proteins from nine species including *Cucumis melo* (BAA36291) and *Hevea brasiliensis* (AAQ63055), while DlHMG2 was grouped in Class B with HMGR proteins from *Taxus* × *media* (AAQ82685) and *Malus* × *domestica* (AAK64657). Interestingly, two HMGR proteins from *A. thaliana* (HMG1; NP_177775 and HMG2; NP_179329), and two from *Malus* × *domestica* (HMG1; AAK64657 and HMG2; ABQ52378) were clustered in two different classes, as for DlHMG1 and DlHMG2, which is consistent with previous observations that the HMGR gene family in plants may contain at least two distinct functional members (Caelles *et al*., 1989). In addition, three HMGR genes from Solanaceous plants [tomato (*Lycopersicon esculentum*; AAB62581), tobacco (*Nicotiana sylvestris*; CAA45181), and potato (*Solanum tuberosum*; P48020)] were also grouped together in Class A. These results indicate that the distribution of HMGR genes in the phylogenetic tree largely reflected the evolutionary relationships between different organisms.

Transcriptional analysis of the DlHMG1 *and* DlHMG2 *genes during fruit development*

As in most other fruit, the growth curves of whole longan fruit and their individual tissues (i.e., pericarp, seed, and aril) display a typical sigmoidal pattern, which can be divided into three phases (Phase I, Phase II, and Phase III; Figure 4). Phase I [0 – 42 d after anthesis (DAA)] was characterised by growth of the pericarp and seed (mainly the seed coat), which accounted for 49.9% and 43.0% of whole fruit growth, respectively. Phase II (42 – 56 DAA) was a period characterised by rapid growth of the cotyledons inside the immature seeds. Growth during this period contributed to 49.5% of all fruit growth, while the arils and pericarp accounted for 24.1% and 26.4%, respectively. Phase III (56 – 81 DAA) was dominated by rapid growth of the aril, which represented 73.3% of all fruit growth, with growth of the pericarp and seeds gradually being suspended.

Northern blot analysis showed that *DlHMG1* gene expression was up-regulated during the early stage of fruit development. High levels of *DlHMG1* transcripts

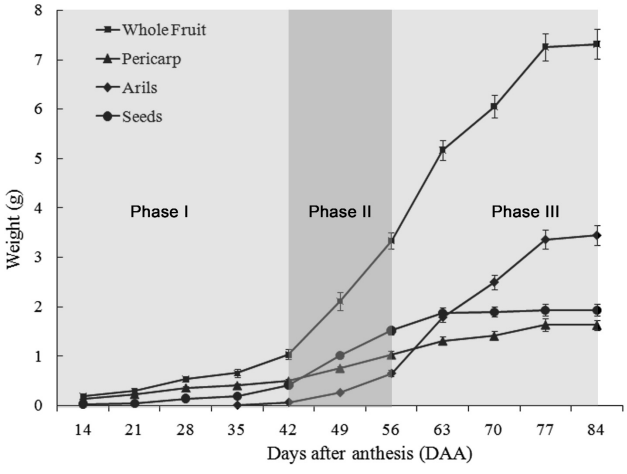

FIG. 4
Changes in individual tissue and total cumulative fresh weights of whole fruit, pericarp, arils, and seeds during longan fruit development. Phases I – III are defined in the text.

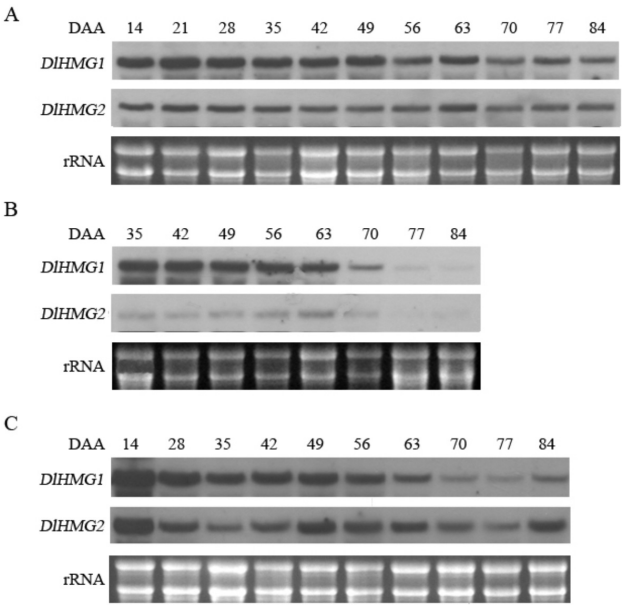

FIG. 5
Changes in the accumulation of *DlHMG1* and *DlHMG2* mRNAs in the pericarp (Panel A), arils (Panel B), and seeds (Panel C) of longan fruit during development. Total RNA (10 µg loaded in each lane) was isolated from the different tissues at the various developmental stages (DAA), and was used for northern blot analysis by hybridisation with *DlHMG1*- or *DlHMG2*-specific DIG-labelled probes. Ethidium bromide-stained rRNA is shown as a loading control.

were detected in Phase I and early in Phase II, but the level started to decrease at 49 DAA and dropped sharply at 63 DAA in all tissues. This dramatic reduction occurred primarily in Phase III, particularly in the arils (Figure 5B) and seeds (Figure 5C). Since rapid cell division was found to occur in Phase I, as well as in part of Phase II in growing longan fruit, the observed high level of *DlHMG1* expression in Phase I and Phase II correlated with active cell division events. Indeed, a similar pattern has also been reported in other plants; for example, tomato (Narita and Grussem, 1989) and melon (Kato-Emori *et al*., 2001) fruit, where *HMGR* gene expression parallelled the stage of rapid cell division during early fruit development. Furthermore, over-expression of a melon *HMGR* gene in transgenic tomato plants caused rapid cell division and an enlarged fruit size (Kobayashi *et al*., 2003), directly demonstrating the role of HMGR in the regulation of cell division and fruit-size during fruit development. Conceivably, *DlHMG1*, like its counterparts in melon and tomato, may be involved in cell division and cell proliferation in longan fruit. However, *DlHMG2* showed a different expression pattern from that observed for *DlHMG1* (Figure 5). *DlHMG2* transcripts in the pericarp remained at a constant level throughout fruit development, but were much less abundant in the early stages compared to those of *DlHMG1* (Figure 5A).

In arils, the level of transcription of *DlHMG2* was similar to that of *DlHMG1* (Figure 5B), with a higher level of expression in Phase I and Phase II, followed by a sharp reduction in Phase III. In seeds, the *DlHMG2* gene showed a periodic pattern, with expression peaks at 14 DAA in Phase I, at 49 – 56 DAA in Phase II, and at 84 DAA in Phase III (Figure 5C). The first two peaks of *DlHMG2* gene expression coincided with vigorous cell division in the seed coat and seed embryo, respectively. The final peak of expression coincided with seed maturation and senescence. Hence, *DlHMG2* may be involved in the regulation of periodic cell division and seed growth during fruit development.

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