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Detection of 3-hydroxy-3-methylglutaryl-coenzyme A reductase protein Cm-HMGR during fruit development in melon (*Cucumis melo* L.)

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Abstract The fruit size of melon (*Cucumis melo* L. *reticulatus*) is determined by the amount of cell proliferation in the pericarp during early fruit development. During this stage, expression and activity of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) gene is required for fruit growth. In this study, we performed a detailed analysis of the correlation between the expression of melon HMGR (Cm-HMGR) protein and cell division in the pericarp. Flow cytometric analysis revealed that the length of the cell division stage was correlated with the fruit size. Western gel blotting and tissue printing illustrated the temporal and spatial accumulation pattern of Cm-HMGR protein during fruit development. The accumulation of Cm-HMGR transiently increased at the beginning of the cell division stage in the pericarp, where active cell division occurred. The amount of Cm-HMGR was correlated with the length of the cell division period. These results strongly suggest that the expression of Cm-HMGR is involved in the determination of melon fruit size by regulating cell division during early fruit development.

Keywords Melon · 3-Hydroxy-3-methylglutaryl coenzyme A reductase · Antibody · Fruit development · Cell division

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Introduction

The regulation of fruit size is an important physiological process during fruit development in higher plants. Early fruit development in higher plants is divided into three distinct phases (Gillaspy et al. 1993). The earliest phase involves ovary development, fertilization, and fruit set. In the second phase, fruit growth is primarily due to cell division, whereas in the third phase, growth occurs by cell expansion. Subsequently, maturation begins. The final size of fruits is essentially determined by the number of pericarp cells defined during the cell division phase (Bohner and Bangerth 1988; Ho 1996; Joubes et al. 1999). Since most studies on fruit development have focused mainly on the ripening phase, little is known about the regulatory mechanisms governing fruit growth.

It has been demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) expression and activity are required for early fruit development in tomato and avocado (Narita and Gruissem 1989; Cowan et al. 1997). HMGR catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A into mevalonic acid, which is a step in the isoprenoid biosynthetic pathway (Chappell 1995a, b; McGarvey and Croteau 1995; Weissenborn et al. 1995). Isoprenoid compounds are involved in various biological processes, including the synthesis of membrane sterols and plant growth regulators (cytokinin, abscisic acid, gibberellins, and brassinosteroids). Previous studies have indicated that the expression of HMGR during early fruit development in tomato and avocado is primarily induced by the metabolic demand for mevalonic acid that is associated with cell division and growth (Narita and Gruissem 1989; Cowan et al. 1995; Jelesko et al. 1999). High HMGR expression and activity were also observed in meristematic tissues and suspension-cultured cells active in cell division and growth (Aoyagi et al. 1993; Enjuto et al. 1994, 1995; Hemmerlin and Bach, 2000). HMGR genes have been isolated from many plant species (Park et al. 1992; Enjuto et al. 1994; Korth et al. 1997; Jain et al. 2000). Plant *HMGR*s constitute small multigene families. Pre-

dicted plant HMGR proteins contain two amino-terminal membrane-spanning and carboxyl-terminal catalytic domains and share a high degree of amino acid sequence-similarity to one another in these domains. In addition, HMGRs are localized in the membrane of the endoplasmic reticulum (Enjuto et al. 1994, Campos and Boronat 1995; Lumbreras et al. 1995; Denbow et al. 1996).

Melon is a good material for studying fruit development for several reasons. First, melon fruit development can be clearly divided into three phases. Second, the structure of the fruit is simple and obvious, and the embryo, flesh, placenta, and seeds are well defined. Third, many genotypes with different phenotypes are available. In order to understand the mechanisms by which the fruit size is regulated, Higashi et al. (1999) used two genotypes, Fuyu A and Natsu 4. Although these genotypes share closely related genetic backgrounds, Fuyu A sets larger fruit than Natsu 4. The results indicated that differences in fruit size between these genotypes are due to the amount of cell proliferation occurring in early fruit development. We also found that a melon HMGR gene (*Cm-HMGR*; Genbank accession no. AB021862) is highly expressed in the early stage of fruit development (Kato-Emori et al. 2001). The expression of *Cm-HMGR* and HMGR activity in Fuyu A and Natsu 4 fruits was proportional to the size of the mature fruit, suggesting that *Cm-HMGR* is involved in fruit growth.

In this study reported here, we attempted to elucidate a correlation between the expression of *Cm-HMGR* and the cell division that contributes to the determination of melon fruit size. We performed detailed analyses of temporal and spatial expression patterns of Cm-HMGR protein during fruit development, in combination with flow cytometric analysis.

Materials and methods

Plant materials

Seeds of two cultivars of muskmelon (*Cucumis melo* L. *reticulatus*), Fuyu A and Natsu 4, were sown and grown in a greenhouse as described (Kato-Emori et al. 2001). Fruit setting after self-pollination was harvested for the experiments on different days after pollination (DAP).

Flow cytometric analysis

Nuclei were prepared from melon fruit pericarp. The pericarp was chopped with a razor blade in nuclear extraction buffer (Partec, Munster, Germany), the samples filtrated through a nylon filter (pore size, 30 μm), and the nuclei in the filtrate stained with 4,6-diamidino-2-phenylindole (DAPI; Partec). Flow cytometric analysis was performed with a Ploidity Analyzer (Partec).

Preparation and purification of anti-Cm-HMGR antibody

A sense primer (forword, 5′-GGAAGCTTAGATCTCTTCTCATCGACAACA-3′) was designed to introduce extra *Hind*III and *Bgl*II restriction sites (underlined) for subsequent subcloning of *Cm-HMGR-CD*. An antisense primer (5′-TCCTCTGCAGTGCCTCACGG-3′) contained *Pst*I sites (underlined). Polymerase chain reaction (PCR) amplification was carried out using a full-length *Cm-HMGR* cDNA isolated from Fuyu A as template, which had been inserted in pBluescriptII SK+ (pBS-HM; Kato-Emori et al. 2001). The amplified PCR fragment was digested with *Hind*III and *Pst*I and then replaced with a *Hind*III-*Pst*I fragment of pBS-HM. The resulting plasmid contains nucleotides encoding Cm-HMGR-CD (the 134–588th residue of Cm-HMGR; the carboxyl terminal region). The Cm-HMGR-CD-encoding fragment was then cut out with *Bgl*II and *Sac*I and cloned in-frame with the amino-terminal hexa-histidie tag of the *Escherichia coli* expression vector, pQE30 (Qiagen, Chatsworth, Calif.). The nucleotide sequence was confirmed. The fusion protein was overexpressed by induction with isopropyl-ß-D-thiogalactopyranoside and affinity-purified using a His-Trap column (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. The sample was fractioned on a 12% sodium dodeycl sulfate (SDS)-polyacrylamide gel (Laemmli 1970), and the gel was stained with Coomassie brilliant blue (Cbb). Gel bands containing the fusion protein were excised and used to raise antiserum in rabbits with the assistance of the Shibayagi Company (Gunma, Japan). To obtain antigen-specific antibody, we recovered the IgG fraction from antiserum using a HiTrap Protein A column (Amersham Pharmacia). The IgG fraction was subsequently cleared by absorption of the cross-reacting IgG to powdered *E. coli* proteins, as described in Sambrook et al. (1989).

Protein preparation, SDS-PAGE and Western gel blotting

We prepared soluble and microsomal membrane proteins from the pericarp of developing melons. Five hundred micrograms of pericarp tissue was homogenized in 1 ml of extraction buffer [100 m*M* Tris-HCl (pH 8.0), 300 m*M* NaCl, 20 m*M* EDTA, 20% (w/v) glycerol, 5 m*M* dithiothreitol (DTT)] with protease inhibitors (1 m*M* phenylmethylsulfonyl fluoride, 0.5 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1 μg ml^{-1} antipain). The homogenate was filtrated through two layers of gauze and centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was recovered and centrifuged at 100,000 g for 30 min at 4 °C. The supernatant thus obtained was used as the soluble fraction. The remaining pellet was resuspended in 50 μl of extraction buffer containing 1% (w/v) Triton X-100, 0.1% (w/v) SDS, and protease inhibitors at the concentrations listed above. The solubilized fraction was used as the microsomal-membrane fraction. The protein concentrations in both the soluble and microsomal-membrane protein samples were determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, Calif.). Protein samples (5 μg) were then mixed with a one-third volume of SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer [250 m*M* Tris-HCl (pH 6.8), 40% glycerol, 8% (w/v) SDS, 100 m*M* DTT, 0.04% (w/v) bromophenol blue] and boiled for 5 min.

The protein samples were separated by SDS-PAGE on 8% (w/v) acrylamide gels. The proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, Mass) as described (Towbin et al. 1979). The membrane was blocked with 1% (w/v) bovine serum albumin in phosphate-buffered saline (8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 140 m*M* NaCl, 2.7 m*M* KCl) containing 0.05% (w/v) Tween 20, and then probed with anti-Cm-HMGR antibody at a 1:1000 dilution. An anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) was used as the secondary antibody at a 1:3000 dilution. Immuno-reactive species were detected with the color development reagent of an Immun-Blot Assay Kit (Bio-Rad).

Western tissue print analysis

Developing melons were cut into slices and placed on PVDF membranes for 5 min. The membranes were then air-dried and subjected to Western tissue print analysis. Immuno-reaction and detection were performed as described above.

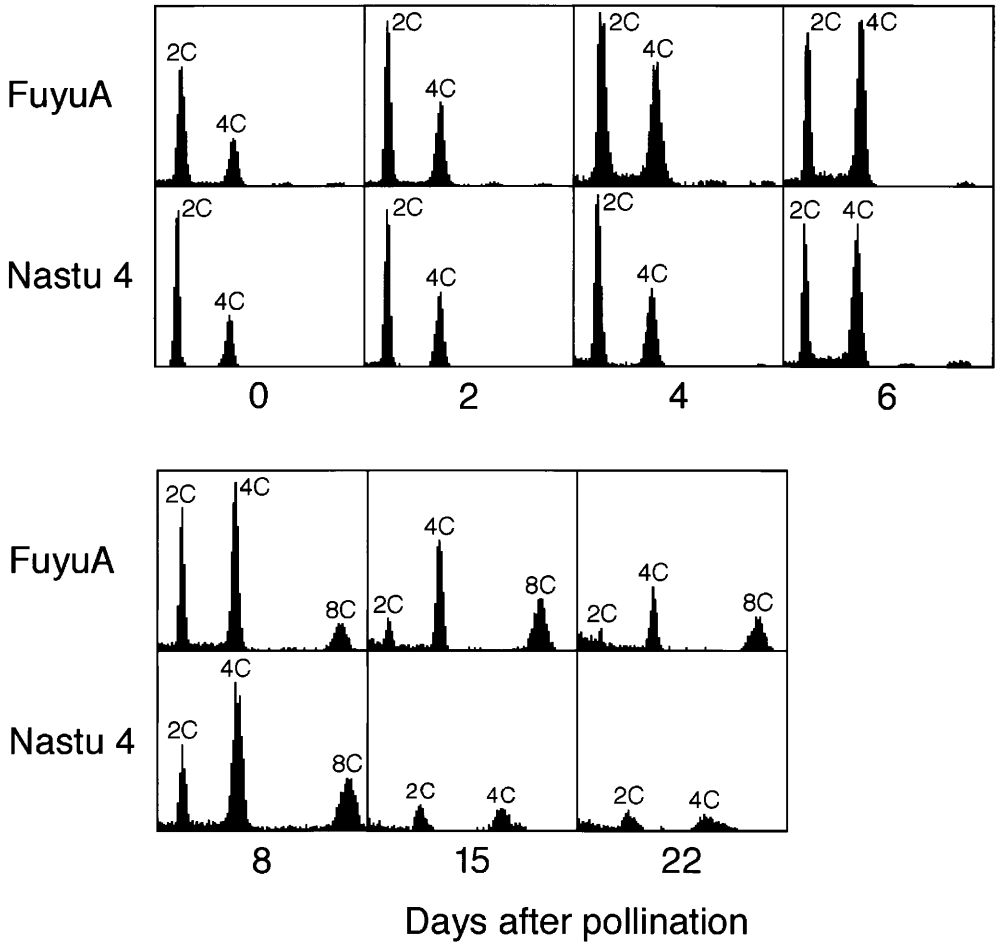

Fig. 1 Flow cytometric analysis of nuclei from the pericarp of developing Fuyu A and Natsu 4 fruits. Nuclei were isolated from Fuyu A and Natsu 4 fruit pericarps on the days after pollination indicated, stained by DAPI, and analyzed by a flow cytometer

Extraction and analysis of phytosterols

Fresh melon fruit (3 g) was extracted and saponified in 5 ml ethanol:methanol (6:1, v/v) containing 10% (w/v) KOH at 70 °C in a shaking water bath for 30 min. The homogenates were filtrated and extracted three times with 15 ml of *n*-hexane. The combined *n*-hexane phase was reduced to dryness by rotary evaporation, and the residue was dissolved in 100 µl of dichloromethane. Each sample (10 µl) was spotted on a silica gel plate (RP-18F254s; Merck, Darmstadt, Germany) and the plate was developed in dichloromethane. The sterols were visualized by spraying with a 3% (w/v) $SbCl_3$ solution in dichloromethane.

Results

Flow cytometric analysis of pericarp cells during fruit development

In order to elucidate the involvement of the cell division in the pericarp in the determination of fruit size, we compared the cell division activity in the pericarp of Fuyu A and Natsu 4 by flow cytometric analysis. The histograms show two major peaks that represent G1- and G2-phase cells with 2C and 4C nuclear DNA contents, respectively (Fig. 1). An increase in the proportion of the 4C peak to the 2C peak indicates the activation of cell division in the tissue, while a decrease indicates the end of cell division. The division of pericarp cells in both Fuyu A and Natsu 4 was activated after pollination. The activation of cell division in Fuyu A rapidly increased within 4 DAP compared to that in Natsu 4. Since a decrease in the propertion of the 4C and 2C peaks indicates the end of cell division growth, cell division in Fuyu A and Natsu 4 was completed within 22 and 15 DAP, respectively. In addition, the appearance of a peak at the 8C DNA level after 8 DAP might be due to the endoreduplication that is accompanied by mitotic arrest. These results revealed that the cell division period in the pericarp of Fuyu A was longer than that in Natsu 4.

Preparation and characterization of antibody against Cm-HMGR

We used the carboxyl terminal region of Cm-HMGR, designated Cm-HMGR-CD, which contains the catalytic domain, to produce antibody against Cm-HMGR protein (Fig. 2A). The carboxyl terminal region of HMGR protein shows extremely high conservation among various plant species. Cm-HMGR-CD was expressed in *E. coli* as a fusion protein. After induction of the recombinant protein, an amplified band with a molecular mass of 55 kDa was observed (Fig. 2B); this is close to the pre-

dicted size of the recombinant protein (51.6 kDa). This affinity-purified protein was used to immunize rabbits. The antigen-specific IgG fraction was purified from the crude antiserum for further analysis. Western blot analysis demonstrated that the antibody specifically recognized the Cm-HMGR-CD antigen (Fig. 2C).

The affinity of the antibody for melon native Cm-HMGR was tested against protein fractions prepared from Fuyu A fruit. Since Cm-HMGR has two putative transmembrane domains at its amino-terminal, Cm-HMGR was expected to be an integral membrane protein. The antibody detected a major protein band at 60 kDa in the microsomal membrane fraction and a minor band at 40 kDa in the soluble fraction (Fig. 2E). The 60-kDa membrane protein represents Cm-HMGR, because the molecular mass is in close agreement with the predicted mass of Cm-HMGR (63.5 kDa). The 40-kDa protein in the soluble fraction might represent a proteolytic fragment from Cm-HMGR. Thus, the antibody was available for the detection of native Cm-HMGR protein in melon.

Expression of Cm-HMGR during fruit development

To investigate the relationship between the expression of Cm-HMGR during fruit development and the activation of cell division in the pericarp, we analyzed the temporal and spatial accumulation pattern of Cm-HMGR protein. Western blot analysis showed two peaks of Cm-HMGR accumulation during Fuyu A fruit development (Fig. 3). The first peak occurred at the beginning of the cell division stage (0–4 DAP), and the second was observed after 22 DAP, after the fruit had enlarged. This suggests that the expression of Cm-HMGR during early developmental stages may be involved in cell division in the pericarp. Therefore, we compared the expression of Cm-HMGR in Fuyu A and Natsu 4 at that stage. The amount of Cm-HMGR in Fuyu A was higher than that in Natsu 4, whereas the accumulation pattern of Cm-HMGR in Fuyu A was similar to that in Natsu 4. These results suggest that the amount of Cm-HMGR protein correlates to the length of the cell division period in the pericarp.

Western tissue printing demonstrated the localization pattern of Cm-HMGR in developing fruit (Fig. 4). This analysis gave results similar to those found by Western blot analysis. Low levels of Cm-HMGR were detected in the placenta and epidermis of the non-enlarged, unpollinated ovule. Immediately after pollination, Cm-HMGR markedly increased in the pericarp and accumulated in the whole fruit. This result supports the involvement of Cm-HMGR expression in the division of pericarp cells.

Changes in sterol content during fruit development

Among the numerous end-products of isoprenoid biosynthesis, membrane sterols are an important factor for cell proliferation. Therefore, we analyzed the composition and content of major sterols during fruit develop-

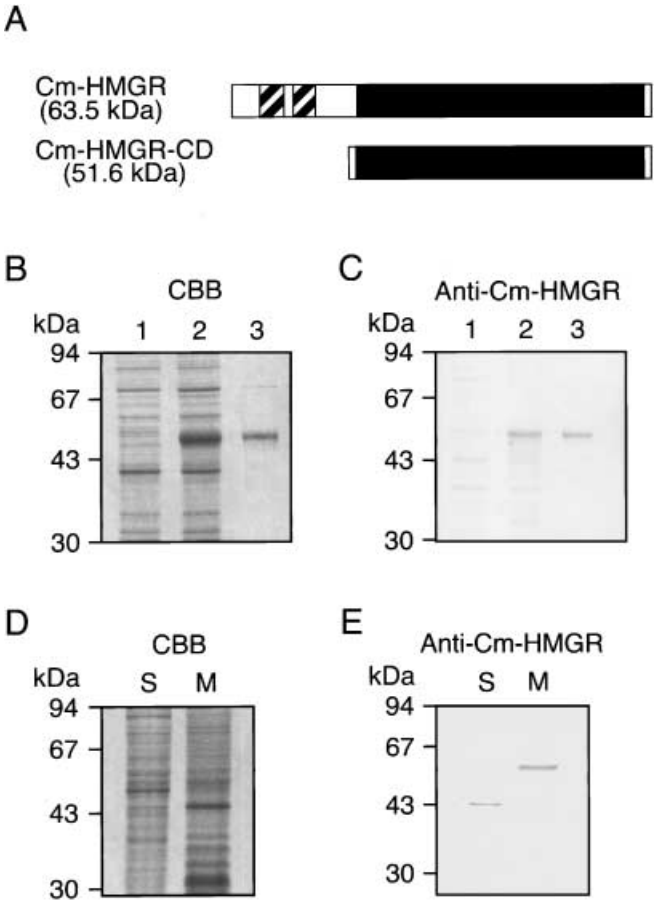

Fig. 2A–E Production of anti-Cm-HMGR antibody and immunodetection of Cm-HMGR in melon fruit. **A** Schematic illustration of Cm-HMGR domain structures and antigen used for antibody production. *Hatched* and *solid boxes* indicate the transmembrane and catalytic domains, respectively. The antigen denoted as Cm-HMGR-CD indicates the 134–588th residue of Cm-HMGR. **B, C** SDS-PAGE gel stained with CBB (**B**) and Western blot analysis of the same gel performed with anti-Cm-HMGR antibody (**C**). *Lane 1* Total proteins from *E. coli* before induction of the expression of Cm-HMGR-CD, *lane 2* total proteins from *E. coli* producing Cm-HMGR-CD, *lane 3* affinity-purified Cm-HMGR-CD. **D, E** Five micrograms of microsomal membrane (*M*) and soluble proteins (*S*) of FuyuA fruit pericarps at 4 days after pollination were separated by SDS-PAGE. Proteins were stained with CBB (**D**) or subjected to Western blot analysis with using anti-Cm-HMGR antibody (**E**)

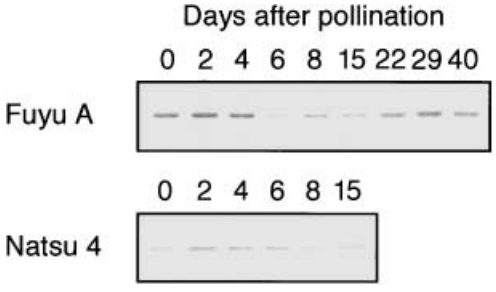

Fig. 3 Stage-specific accumulation of Cm-HMGR protein during fruit development of Fuyu A and Natsu 4. Microsomal membrane proteins were prepared from Fuyu A and Natsu 4 fruit pericarps on the days after pollination indicated. The proteins were separated by SDS-PAGE (5 μg of protein per lane) and subjected to Western blot analysis with anti-Cm-HMGR antibody

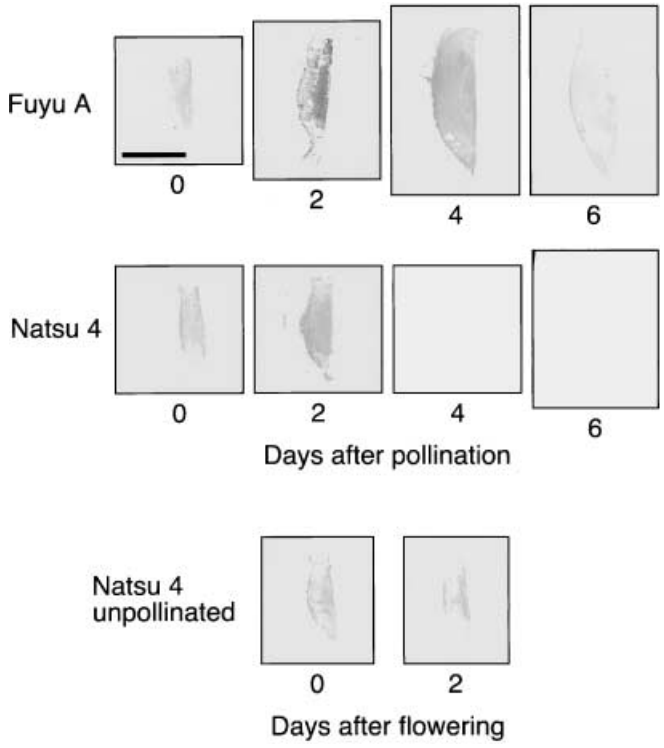

Fig. 4 Tissue-specific accumulation of Cm-HMGR protein during fruit development of Fuyu A and Natsu 4. Transverse sections from the centers of Fuyu A and Natsu 4 fruit were placed on PVDF membranes for 5 min. The membranes were then subjected to Western tissue print analysis with anti-Cm-HMGR antibody. The *top* of each panel indicates the calyx side. *Bar*: 1 cm

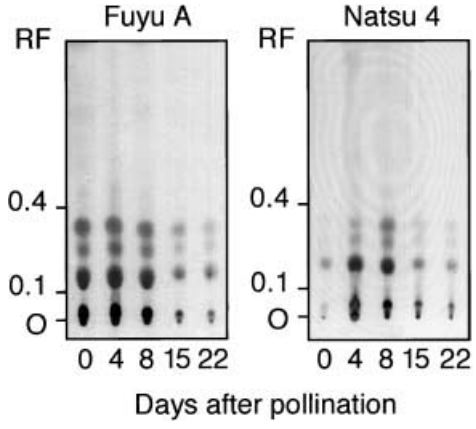

Fig. 5 Sterol content and composition during Fuyu A and Natsu 4 fruit development. Sterols were extracted from Fuyu A and Natsu 4 fruits, separated by thin-layer chromatography on a silica gel plate developed with dichloromethane and stained with $SbCl_3$. To allow for direct comparison, all samples (3 g of fresh weight) were extracted in 5 ml of 10% KOH in an ethanol:methanol solution, and equal aliquots were spotted on a plate. Sterols migrated between RF 0.1 and 0.4. *O* Origin

ment by thin layer chromatography. The sterol content was correlated with the expression of Cm-HMGR and the final fruit size. High sterol levels were observed during the cell division stage (0–8 DAP); these levels decreased after 15 DAP (Fig. 5). The total sterol content in Fuyu A was higher than that in Natsu 4. In contrast, the composition of sterols did not differ between Fuyu A and Natsu 4.

Discussion

The flow cytometric analysis revealed that the length of the cell division phase affects the final fruit size during melon fruit development (Fig. 1). The transition from cell division phase to cell expansion phase was identified by a decrease in the total number of nuclei and by the appearance of an 8C DNA peak. The decrease in nuclear numbers occurs with the expansion of pericarp cells, which are initially small and tightly compressed during the cell division phase (Higashi et al. 1999). The increase in the 8C DNA peak is due to endoreduplication (D'Amato 1984). The multiplication of nuclear ploidity observed in tomato fruit seems to be associated with cell expansion in the pericarp and gel tissues (Joubes et al. 1999). Histological observation of melons suggested that the estimated cell number in fruit is positively correlated with the final fruit size (Higashi et al. 1999). In tomato and avocado, the cell number in the pericarp is a major determinant of fruit sizes (Cowan et al. 1997; Joubes et al. 1999; Frary et al. 2000). Our result is consistent with these observations and more directly indicates that the length of the cell division phase in the pericarp is involved in the determination of the final size of melon fruit.

Western analysis demonstrated that the expression of Cm-HMGR during early fruit development is closely correlated with cell division in the pericarp, which determines the final size of melon fruit. The Cm-HMGR content increased markedly in the pericarp, where frequent cell division occurs, at the beginning of the cell division phase (Figs. 3, 4). The expression pattern of Cm-HMGR was similar to that of the tomato HMG1 gene (Narita and Gruissem 1989; Jelesko et al. 1999). The expression of *HMG1* in tomato fruits is associated with cell proliferation in the pericarp, since high expression of *HMG1* and other plant *HMGR*s is also observed in meristematic tissue and cultured cells where active cell proliferation is occurring (Aoyagi et al. 1993; Enjuto et al. 1994, 1995; Jelesko et al. 1999). The comparison of Cm-HMGR expression between Fuyu A and Natsu 4 supports the correlation between Cm-HMGR expression and cell division in the pericarp (Fig. 3). The amount of Cm-HMGR protein was proportional to the length of the cell division period. In avocado, HMGR activity is also associated with differences in fruit size (Cowan et al. 1997). In addition, the application of a competitive inhibitor of HMGR, mevastatin, down-regulates avocado fruit growth by reducing the number of cells (Cowan et al. 1997). Therefore, the amount of Cm-HMGR protein is involved in the determination of fruit size by regulating the length of the cell division period.

HMGR is induced by the metabolic demand for mevalonic acid, which is essential for the process of cell

division (Jelesko et al. 1999). Although tomato *HMG1* does show an expression pattern similar to those of genes that do control the cell-division cycle – e.g., cyclin-dependent kinases and cyclins (Martinez et al. 1992; Ferreira et al. 1994; Feiler et al. 1995; Joubes et al. 1999) – HMGR may not have a direct regulatory role in the progression of the cell cycle. The sterol analysis of developing melon fruits showed that the sterol content increased with the expression of Cm-HMGR at the cell division phase during early fruit development and was correlated with the final fruit size (Fig. 5). Sterols are important for cell proliferation as membrane components that regulate membrane fluidity and permeability (Hartmann 1998). Therefore, the elevated Cm-HMGR level may increase the pool of mevalonic acid, thus promoting the supply of membrane sterols, which in turn enables the cell division phase to be extended during early fruit development.

The expression of *Cm-HMGR* during late fruit development showed a similar pattern to that of tomato *HMG2*, which is induced by metabolic changes associated with fruit ripening. However, we previously demonstrated that accumulation of *Cm-HMGR* transcripts occurred during early fruit development and ripening in melon, whereas HMGR activity was associated only with early fruit development (Kato-Emori et al. 2001). Western blot analysis revealed two peaks of Cm-HMGR protein accumulation, consistent with the accumulation of its transcripts (Fig. 3). Therefore, HMGR activity must be controlled at the protein level during the ripening stage, although the reason for the accumulation of inactive Cm-HMGR during fruit maturation is unclear. Plant HMGRs are regulated not only transcriptionally, but also translationally and post-translationally (Oosterhaven et al. 1993; Stermer et al. 1994; Hemmerlin and Bach 1998). A possible cause of the inactivation of Cm-HMGR may be reversible phosphorylation (Stermer et al. 1994). Plant HMGR is inactivated via phosphorylation by a specific HMGR kinase and activated by a phosphatase (MacIntosh et al. 1992; Dale et al. 1995). Alternatively, Cm-HMGR might be inactivated by a limitation of its cofactors. HMGR requires calcium as a cofactor for its activity (Stermer et al. 1994). In banana, a rapid decline in the levels of ascorbate and iron causes immediate inactivation of 1-aminocyclopropene-1-carboxylic acid oxidase during the late ripening stages (Liu et al. 1999).

This study also suggested that proteolytic degradation might be involved in the regulation of Cm-HMGR expression. Western blot analysis showed that a 40-kDa protein in the soluble fraction cross-reacted with anti-Cm-HMGR antibody (Fig. 2E). This protein band may contain a proteolytic fragment of Cm-HMGR that lacks the transmembrane domains. A recent study revealed that the degradation of HMGR serves to help regulate its activity in potato (Korth et al. 2000). The degradation of HMGR is controlled by cysteine proteases, since cysteine protease inhibitors increases recovery of HMGR protein and its activity (Stermer et al. 1994; Korth et al. 2000).

Plant HMGRs are controlled by complicated developmental and environmental signals because HMGR is a key enzyme of the isoprenoid pathway of which end products have diverse important functions in various physiological responses of plants (Stermer et al. 1994). In the study reported here, we forward the possibility that *Cm-HMGR* is regulated at the levels of enzyme activity and protein degradation during melon fruit development, as well as at the transcriptional level. The use of melon fruit as experimental material will provide new insights into the regulatory mechanisms of the expression and activity of plant HMGRs. We are now taking a transgenic approach to evaluate the regulatory role of *Cm-HMGR* in the determination of fruit size.

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