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Original Paper

Molecular and genetic characterization of transgenic tomato expressing 3-hydroxy-3-methylglutaryl coenzyme A reductase

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Abstract Mevalonic acid (MVA) pathway, in parallel with methylerythritol phosphate (MEP) pathway, produces precursor metabolites for isoprenoids, and affect fruit development in plants. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is supposed to be a key enzyme in the MVA pathway. To understand the role of HMGR in fruit development, we previously generated transgenic tomato (*Solanum lycopersicum*) expressing a melon *HMGR* gene (*CmHMGR*) and observed increased fruit size in these plants. To further examine this effect, we performed molecular and genetic characterization of the transgenic tomato line in the T_4 generation. The line showed stable expression of *CmHMGR* mRNA and protein, an effect that could lead to the increase in fruit weight observed, which exceeded 20%. Interestingly, the *CmHMGR* mRNA was highly expressed during tomato fruit development, whereas expression of the endogenous *HMGRs* (*SlHMGR1*, *SlHMGR2*, and *SlHMGR3*) was lower than in the wild type, suggesting the presence of a regulatory mechanism at the transcriptional level, as in mammalian systems. A preliminary analysis using cDNA macroarray filters was performed, and genes showing more than 2.5-fold differences in expression between transgenic and wild-type plants were identified. Most of the genes involved in isoprenoid biosynthesis did not show significantly different transcription levels, but 121 annotated genes and 152 genes of unknown function were found to be differentially expressed. These results demonstrate that the transgenic tomato line expressing the *HMGR* gene is genetically stable and could be used as a comprehensive material to elucidate the roles of HMGRs in tomato fruit development.

Key words: DNA array, Fruit size, HMG-CoA reductase, *Solanum lycopersicum*.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) converts HMG-CoA into mevalonic acid (MVA) and supposed to play a key role in the MVA pathway (Hemmerlin et al. 2003). This pathway produces a tremendous variety of compounds that are essential for plants, such as phytosterols involved in cell structure (Ha et al. 2001), phytohormones (Cowan et al. 1997), defense and stress response components (Yang et al. 1991; Choi et al. 1992), and secondary metabolites (Stermer et al. 1994; Lichtenthaler et al. 1997, 2003; Schaller 2003). Compounds derived from the MVA pathway contribute to the growth of various species of higher plants in diverse environments.

In the last decade, tomato has been utilized as a model system for studying fruit development (Narita and Gruissem 1989; Gillaspy et al. 1993). Cell division and expansion are considered two distinct phases of tomato fruit development (Cong et al, 2002) and are the major factors that define fruit size. The earliest event involves active cell division before and after fertilization. Following fertilization, active cell division lasts for 6–15 days (Bohner and Bangerth 1988; Kobayashi et al. 2003; Bertin et al. 2003). Pollination appears to be an important stimulus for the subsequent fruit development. As cell division abates, the cell expansion stage begins. Enlargement of individual cells occurs, which rapidly increases fruit volume. After full enlargement of the fruit, ripening and maturation take place.

HMGR has been demonstrated to be essential for normal fruit development in studies using the HMGR inhibitors mevastatin, mevinolin, and arachidonic acid, or using the cultivars that expressed different levels of HMGR (Narita and Gruissem 1989; Cowan et al. 1997; Rodríguez-Concepción and Gruissem 1999; Kato-Emori et al. 2001). Investigations during the course of fruit ontogeny revealed the contribution of HMGR to cell proliferation in avocado and melon pericarp (Cowan et al. 1997; Rodríguez-Concepción and Gruissem 1999; Higashi et al. 1999; Kobayashi et al. 2002).

In addition to these studies, transgenic plants have been used to examine the roles of *HMGRs* in fruit development. Using *Agrobacterium*-mediated

transformation, we generated transgenic tomato plants that constitutively express a heterologous *HMGR* gene from melon, *CmHMGR* (Kobayashi et al. 2003). Among the T_0 and T_1 generations of the transgenic lines, lines expressing moderate levels of the *CmHMGR* mRNA showed a significant increase in fruit size that resulted from a prolonged cell division period after pollination. These transgenic tomato lines could be useful for understanding functional role of HMGR in fruit enlargement.

The purpose of this study was to perform molecular and genetic characterization of transgenic tomato expressing the *CmHMGR* gene. To this end, we established a genetically stable line expressing *CmHMGR* obtained in our previous study, by self-pollinating the line through several generations (Kobayashi et al. 2003). In addition, we carried out cDNA macroarray analysis using the stable line to better understand the role of HMGR in tomato fruit enlargement and development.

Materials and methods

Plant material and growth conditions

The transgenic tomato line CD-25 (T_4 generation) expressing the melon *HMGR* gene (*CmHMGR*), which was generated in our previous study (Kobayashi et al. 2003), and the wild-type cultivar Moneymaker were used. Seeds were surface-sterilized, sown on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% sucrose, and grown in a growth chamber at 25°C. Seedlings were transplanted into pots and placed in a glass house for three weeks. Plants were grown under standard greenhouse conditions. The flowers were artificially pollinated by vibration, and the number of fruits in each bunch was restricted to five.

Morphological analysis of seedlings and fruits

Seeds of the transformants and wild-type were sown on MS medium. The lengths of hypocotyls and roots were measured 15 d after sowing. To generate a cross between the wild-type and the transformant, pollen of the transformant was applied to wild-type flowers that had been emasculated prior to anthesis. The F_1 seeds obtained from these fruits were sown on commercial soil (Kureha, Tokyo, Japan) and the phenotypes were observed. Fruits were harvested winter 2005 and spring 2006. The diameters, lengths, and hundred-seed weights of these fruits were measured and the fruit indices were calculated.

Total RNA preparation and RT-PCR

Total RNA from 6 day-post-pollination (DPP) tomato fruits was isolated using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNAs from 30DPP, coloring stage, and maturation stage tomato fruits were extracted as described by Yamamoto (2005). Reverse transcription was performed with 1 μg of total RNA as a template and SuperScript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). Fragments of 707, 891, 804, and 301 bp containing the coding regions of *CmHMGR* (accession number AB021862), *SlHMGR1* (accession number L40938), *SlHMGR2* (accession number M63642), and *SlHMGR3* (Contig15128), respectively, were amplified by RT-PCR using the following primer sets: (forward) 5′-GGTTCTGTTCCTTCGTACT-3′ and (reverse) 5′-TATTACTGCCTCACAGACCACA-3′, (forward) 5′-CCACACAAACCATCACTGCT-3′ and (reverse) 5′-GTCA-TGCCATCTCTGAGCAA-3′, (forward) 5′-TGCTCTTGTG-GAGCTGAA-3′ and (reverse) 5′-ATAGCTGACATGAGGG-ATAG-3′, and (forward) 5′-ACAGCTTCAACACTCAC-3′ and (reverse) 5′-GGAGTGGAATTCCATTACGG-3′. The hypothetical sequence for *SlHMGR3* was obtained from the tomato database MiBASE (http://www.kazusa.or.jp/jsol/microtom/index.html) (Yano et al. 2006), based on the sequence of an EST with strong sequence similarity (84%) to potato (*Solanum tuberosum*) *HMG3.3* (accession number Q41438). The sequence of the amplified fragment probably representing *SlHMGR3* was confirmed by sequence analysis.

RT-PCR-Southern analysis

PCR reactions were performed using 12 cycles for *CmHMGR-CD*, *SlHMGR1*, and *SlHMGR2* and 20 cycles for *SlHMGR3*. The PCR-amplified fragments were electrophoresed in 1% agarose gels, transferred to positively charged nitrocellulose membrane (Hybond N^+, GE Healthcare UK Ltd., Tokyo, Japan) by capillary transfer, and subjected to hybridization. Probes were amplified with the *CmHMGR-CD*, *SlHMGR1*, *SlHMGR2*, and *SlHMGR3* primer sets listed above. Labeling and hybridization to the membrane were performed with AlkPhos Direct (GE Healthcare UK Ltd., Tokyo, Japan) according to the manufacturer's instructions with a high-stringency wash at 55°C.

Protein preparation and immunoblot analysis

Protein samples were prepared as described (Kobayashi et al. 2003) from 15-day-old seedlings. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, Cal.), and 5-μg samples were used for SDS-polyacrylamide gel electrophoresis in 12% (w/v) acrylamide gels. The proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Mass.). Immunoblot analysis was performed with anti-CmHMGR antiserum, which dose not cross-hybridize to SlHMGRs, at a 1 : 1000 dilution and Goat Anti-Rabbit IgG HRP Conjugate (Bio-Rad, Hercules, Cal.). Bound antibody was visualized with a Peroxidase Stain Kit for Immunoblotting (Nacalai Tesque, Kyoto, Japan).

Hybridization of cDNA macroarray filters

The cDNA macroarrays were contributed by the Japan Solanaceae Consortium (JSOL). Based on expression sequence tags obtained from cDNA library from miniature cultivar tomato "Micro-Tom", 12,164 unique cDNAs from leaf and fruit were spotted on nylon membrane, and used for macroarray hybridization (Yano et al. 2006). Five micrograms of total RNAs isolated from four fruit developmental stages were reverse-transcribed and labeled with [α-^{33}P]dCTP. The probes were purified with a QIAquick PCR Purification Kit (Qiagen,

Tokyo, Japan). Subsequently, the labeled probes were hybridized with the cDNA macroarray filters for 16 h at 55°C. The signal intensities were gauged with a BAS-1800II (Fujifilm, Tokyo, Japan) image reader and ArrayGauge ver. 1.31 (Fujifilm, Tokyo, Japan) software.

Results

Stable expression of CmHMGR-CD in T_4 transgenic plants

Transgenic tomato lines were generated by *Agrobacterium*-mediated transformation of CaMV35S::*CmHMGR* (Figure 1A), and one line, CD-25, was chosen for subsequent experiments because the other lines either did not bear fruit or the expression of the transgene was not significantly elevated in the fruits (Kobayashi et al. 2003). The CD-25 line was maintained by self-pollination. A *CmHMGR-CD* homozygous line was selected, and the T_4 generation was used for subsequent experiments. The stable expression of the *CmHMGR-CD* mRNA and the resulting protein in tomato seedlings were detected using a specific primer set and anti-CmHMGR antiserum, respectively (Figure 1B), indicating that the transgene was inherited in a genetically stable manner.

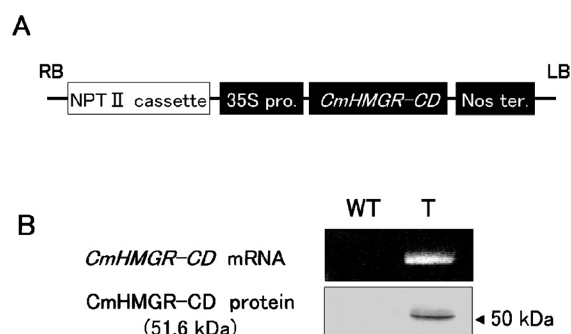

Figure 1. The structure of the transgene and expression analysis in T_4 generation tomato seedlings. (A) *CmHMGR-CD* was introduced in tomato by *Agrobacterium*-mediated transformation. *CmHMGR-CD* is driven with the constitutive expression promoter, Cauliflower Mosaic Virus 35S promoter. (B) A set of *CmHMGR-CD* specific primer and anti-CmHMGR were generated. The expression of the transgene was detected by RT-PCR analysis (upper) and Western blot analysis (lower).

Shortened hypocotyls and roots in transgenic seedlings and dominance of the transgene

Wild-type and T_4 transgenic plant seeds were grown on MS medium and the lengths of hypocotyls and roots of 15-day-old seedlings were measured. The lengths of hypocotyls and roots of the transgenic seedlings were approximately half those of the wild-type (Figure 2A). The dominance of the transgene was tested by applying pollen from homozygous T_3 transgenic plants to wild-type flowers whose stamens had been removed prior to anthesis. The resulting hybrid seedlings showed the same phenotype as the transgenic homozygous seedlings (Figure 2B), indicating that the transgene acts in a dominant manner.

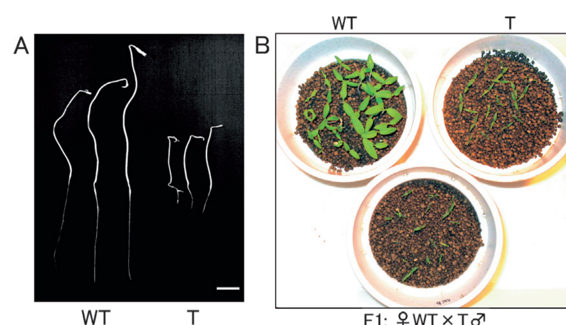

Figure 2. Transgene caused the dwarf phenotype of seedlings and the dominancy of transgene. (A) Transformants showed about half length of hypocotyl and root. The seeds were surface-sterilized and sown on MS medium containing 3% sucrose. White bar indicates 1 cm. (B) Cross between wild-type and the transformant revealed that the dwarf phenotype is due to the transgene. (♀) representing pistil supplier, and (♂) pollen.

Effect of the transgene on tomato fruit weight

Wild-type and transgenic tomato plants were grown in a semi-containment greenhouse during two growth periods, winter 2005 and spring 2006. Because the growth of the transgenic plants was retarded by about two weeks in the seedling stage, as shown in Figure 2B, the transgenic seeds were sown two weeks earlier than the wild-type seeds in order to synchronize the flowering time. To examine the fruit morphology, 79 wild-type fruits and 58 transgenic fruits were harvested in winter 2005 and 94 wild-type fruits and 63 transgenic fruits were harvested in spring 2006. In contrast to the seedling phenotype, both the length and the diameter of the transformant fruits were significantly increased, and their fresh weight was more than 20% greater than that of the wild-type (Table 1).

Expression analysis of the transgene CmHMGR-CD and the endogenous SlHMGRs in tomato fruits

To investigate an influence of CmHMGR overexpression on expression levels of tomato endogenous HMGR genes, specific primer sets for each gene were synthesized and mRNA levels were determined by RT-PCR-Southern analysis. As previously reported (Gillaspy et al. 1993), *SlHMGR1* was strongly expressed in young fruit, and its expression gradually decreased until the onset of ripening, after which it increased slightly (Figure 3). In contrast to the results of Gillaspy et al. (1993), the *SlHMGR2* expression pattern was similar to that of *SlHMGR1* (Figure 3). In addition, *SlHMGR3* mRNA accumulated in 30DPP and maturation-stage fruits (Figure 3). Although *CmHMGR-CD* was strongly

Table 1. Morphological analysis on fruits of transgenic and wild-type plants, harvested in the spring of 2005 and the winter 2006.

	Hundred-seed weight (mg)	Length (mm)	Diameter (mm)	Fruit index	Fresh weight (g)	FW of T/ FW of WT (%)
2006 winter						
WT	358 a	44.7 a	52.0 a	0.86 a	67.8 a	100
T	349 a	48.3 b	55.1 b	0.88 a	83.7 b	124
2005 spring						
WT	348 a	39.4 a	45.7 a	0.86 a	53.9 a	100
T	356 a	42.5 b	49.2 b	0.86 a	64.8 b	120

More than 55 fruits in maturation stage were harvested in each period and used for the analysis. Different characters indicate statistically significant differences ($P<0.05$), calculated using the Student's *t*-test.

Table 2. Transcriptional analysis of the genes involved in isoprenoid biosynthesis with cDNA macroarray using the transgenic and the wild-type plants.

Annotation[b]	Fold change T/WT[a]			
	6DPP[c]	30DPP	Orange	Red
isopentenyl pyrophosphate isomerase	0.786	1.276	0.726	0.720
geranyl pyrophosphate synthase large subunit	0.945	0.589	0.372	0.668
geranylgeranyl pyrophosphatase related protein	0.823	1.092	1.25	1.01
geranylgeranyl reductase	n.d.d	0.779	1.241	0.997
fernesyltransferase alpha subunit	0.803	1.404	0.661	0.823
fernesyltransferase subunit B	0.928	0.996	n.d.	n.d.
squalene monooxygenase	0.888	1.158	1.044	0.967
11-beta-hydroxysteroid dehydrogenase	1.091	1.027	0.910	1.021
delta-7-sterol-C5-desaturase	0.655	n.d.	n.d.	n.d.
S-adenosyl-methionine-sterol-*C*-methyltransferase	0.992	0.947	0.968	0.800
3-beta-hydroxysteroid dehydorogenase	0.662	n.d.	n.d.	n.d.

[a] Mean ratio of the normalized data between the transformant (T) and wild-type (WT). [b] Putative function according to NCBI, TIGR or TAIR EST databases. [c] DPP: day-post-pollination. [d] Not detected in both wild-type and transformant.

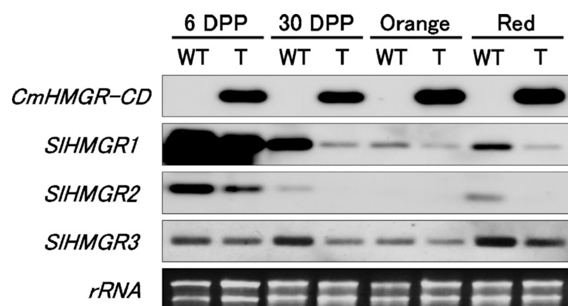

Figure 3. Analysis of *CmHMGR-CD*, *SlHMGR1*, *SlHMGR2* and *SlHMGR3* transcripts by RT-PCR-Southern blotting with sets of gene specific primer. For *CmHMGR-CD*, *SlHMGR1* and *SlHMGR2*, 12 cycles of PCR have performed, and 20 cycles for *SlHMGR3*.

expressed in transgenic tomato fruits at all four developmental stages, the expression levels of the tomato *HMGRs* were slightly decreased in all stages (Figure 3), suggesting the existence of a mechanism that regulates the endogenous *HMGRs*.

Transcription analysis of genes involved in isoprenoid biosynthesis

To investigate the influence of *CmHMGR-CD* overexpression on the genes that catalyze reactions in the later steps in the MVA pathway, cDNA macroarray analysis was performed. Eleven spots possibly representing genes involved in the MVA pathway were selected, and their mRNA levels in the wild-type and the transformant were compared (Table 2). A gene encoding the geranyl pyrophosphate (GPP) synthase large subunit was significantly suppressed from the 30DPP stage to the red stage, but no obvious changes were observed in the expression of the ten other genes, from early fruit development to the red stage.

Comprehensive analysis of transcript levels in transformant fruits

Next, we searched for tomato genes that showed significantly different expression levels in *CmHMGR* transformants. By comparing macroarray data of the wild-type and the transformant, we identified genes whose signal intensities differed more than 2.5-fold. The genes were classified according to putative functions (Table 3). In the 6DPP stage, 36 annotated genes, and 42 genes of unknown function or with no match in the database showed significantly different expression levels. For example, the expression of bax inhibitor, polygalacturonase, cellulose synthase, 2-isopropylmalate synthase A, thioredoxin h and alchol acyl transferase were more than 2.7-fold greater in the transformant, and the expression of alchol dehydogenase-2 was only one third of that of the wild type. Similarly, 28, 43, and 14 annotated genes, and 29, 58, and 23 genes of unknown

Table 3. Comprehensive transcriptional analysis of the differentially expressed genes with cDNA macroarray using the transgenic and the wild-type plants.

Clone ID[a]	Annotation[b]	Fold change T/WT[c]	Stage[d]
Phytohormon-related genes			
LA12AG11	ethylene response factor 2	0.4	30DPP
Cell cycle and DNA processing			
LA13AF11	replication factor C	5.29	30DPP
FA56AA04	cell division protein FtsH-like	0.39	Orange
Cell fate: growth regulators/regulate of cell size			
FA25DA07	Bax inhibitor	2.73	6DPP
FA52AH01	LMBR1 integral membrane protein	0.23	30DPP
FA14CH11	MTN3 protein	0.22	30DPP
FB08AC01	floral homeotic protein TAG1	27.3	Red
Cell rescue defense, virulence and detoxification			
FA25CF08	prohibitin 1-like protein	0.37	6DPP
FA30BA05	flavin-containing monooxygenase family protein	12.7	30DPP
LA28BG03	glutathione reductase	0.19	30DPP
LA28DG08	metallothionein-like protein	3.85	Orange
FA16CF11	pathogenesis-related protein PR P23	0.38	Orange
FB14CD01	antifungal protein	0.25	Orange
FB18BA12	defense-signaling glycopeptide hormone precursor	0.18	Orange
Stress responsive			
FA32CH04	heat shock transcription factor family protein	0.17	30DPP
LB10DA07	UMUC-like DNA repair family protein	3.31	Orange
FA16BA10	heat shock protein 83	0.32	Orange
LB04BG06	multiple stress-responsive zinc-finger protein	0.09	Orange
Cellular communication/siganl transduction			
FA31AD06	receptor-like protein kinase	4.72	6DPP
FA25DC01	shaggy like protein kinase	0.01	6DPP
LA25BF01	leucine-rich repeat transmembrane protein kinase	5.92	30DPP
LA19AF01	inositol monophosphatase 3	5.34	30DPP
FB06BC08	receptor protein kinase	2.58	30DPP
FA24BH08	bystin	0.26	30DPP
FA31CA01	serine/threonine protein kinase	0.37	Orange
Localization			
FA04CA01	beta-adaptin-like protein B	6.12	6DPP
Cellular organization, cell wall			
LA25AA07	pectinesterase family protein	10.2	6DPP
FA16DC10	clathrin coat assembly protein AP50	4.87	6DPP
FB12CA07	cellulose synthase	3.7	6DPP
FA03CD03	Arabidopsis dynamin-like protein ADL2	2.95	6DPP
LB11DG05	proline-rich proteoglycan 2	5.28	Orange
Respiration			
FA23AD10	2-isopropylmalate synthase A	2.99	6DPP
FA42AD03	induced stolon tip protein NAP1Ps	3.39	30DPP
FA30CD01	cytochrome c1 precursor	0.31	Orange
FA17AE06	mitochondrial NAD-dependent malate dehydrogenase	0.24	Orange
FA23CA06	NAD-dependent malic enzyme 59 kDa isoform, mitochondrial precursor	51.6	Red
Photosynthesis			
FB04BC08	thioredoxin h	3.85	6DPP
LB01AB07	photosystem I reaction center subunit X psaK	2.6	6DPP
LC19BD11	Tic62 protein	0.37	6DPP
LC04CE03	RuBisCO activase 1	0.3	6DPP
LA22DH03	chlorophyll *a*/*b*-binding protein type I	0.19	30DPP
FB14AD06	quinone reductase family protein	17.2	Orange
LA26CE04	rubisco expression protein	5.02	Orange
LC04DE07	NADH2 dehydrogenase	2.93	Orange
FB05DB06	NADH-ubiquinone oxidoreductase 12 kD subunit	0.34	Red
Carbohydrate metabolism			
FA34BG07	polygalacturonase	11.2	6DPP
LB04AF08	UDP-glucose:sterol 3-O-glucosyltransferase	3.17	6DPP
FA24CC06	UDP-glucuronate decarboxylase 1	0.22	6DPP
FA04BH12	UDP-glycosyltransferase 89B2	0.06	6DPP
FB15AE01	alpha-glucosidase	21.1	Orange
FA22AE08	glucose-1-phosphate adenylyltransferase small subunit	5.37	Orange
LC12DB07	starch synthase	4.4	Orange
FA32DG07	alpha-glucosidase	3.19	Orange
FA27CG06	xylulose kinase	0.34	Orange
FA24BH02	UDP-glucose glucosyltransferase-like protein	0.23	Orange
FB09AA05	soluble starch synthase	0.15	Orange
FA25AH12	pyrophosphate-fructose-6-phosphate 1-phosphotransferase-like protein1	4.6	Red

(Table continues on following page.)

Table 3. (Continued from previous page.)

Clone ID[a]	Annotation[b]	Fold change T/WT[c]	Stage[d]
FB09BB05	fructose-1,6-bisphosphatase	0.38	Red
LC14AD04	phosphoenolpyruvate carboxylase	0.37	Red
Nucleotide metabolism			
LA14AC03	GTP cyclohydrolase I	0.27	6DPP
FA25BH05	GTP-binding protein	0.07	6DPP
FA12DF01	GTP-binding family protein	14.9	Orange
Amino acid metablism			
FA29BH06	beta-cyanoalanine synthase	3.7	6DPP
FA29BE02	5-phosphoribosyl-1-pyrophosphate amidotransferase	0.35	30DPP
LA28AF11	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein	0.2	30DPP
FA35CF10	methionine aminopeptidase	0.17	30DPP
LC08BF11	formamidase	0.14	30DPP
FA02CH05	NADH dehydrogenase	12.9	Orange
LA20CD07	low molecular weight phosphotyrosine protein phosphatase family protein	11.7	Orange
LB01DF12	acireductone dioxygenase (ARD/ARD′) family protein	8.3	Orange
FA32CF07	adenylosuccinate synthase	0.27	Orange
LA27BC11	cystathionine beta-lyase	0.4	Red
Lipid, fatty acid and sterol metabolism			
FA21BG08	oxysterol-binding family protein	6.96	30DPP
LB04AG08	biotin synthase	2.89	30DPP
LA26BF11	*S*-adenosyl-methionine-sterol-*C*-methyltransferase homolog	2.58	Orange
FA10AB11	ras-related GTP-binding protein	0.3	Orange
FA21BG08	oxysterol-binding family protein	0.29	Orange
FA34CA01	beta-ketoacyl-ACP reductase	0.09	Orange
FA06AF03	phosphatidate cytidylyltransferase	0.35	Red
LB02CE08	sulfolipid synthase	0.26	Red
Fermentatio			
FA03CG01	alcohol acyl transferase	2.71	6DPP
FA15DG02	alcohol dehydrogenase-2	0.32	6DPP
Protein synthesis and degradation			
FB09CG07	ubiquitin / ribosomal protein CEP52	2.92	6DPP
LB11AC04	ribosomal protein S27	2.73	6DPP
LB02CB10	ribosomal protein L24	5.14	30DPP
LB11BG01	40S ribosomal protein S16	3.54	30DPP
FB12DC07	ribosomal protein L5	2.73	30DPP
LC06AE04	subtilisin-like protease	0.11	30DPP
FA42CA01	26S proteasome non-ATPase regulatory subunit 3	5.98	Orange
FA04BH10	translation initiation factor eIF3	4.63	Orange
LC16CB05	ribosomal protein S26	0.05	Orange
LB14CC05	ribosomal protein S19	10.7	Red
FA15AB12	translation elongation factor eEF-1 alpha chain	2.88	Red
FA35DB09	ubiquitin-specific protease 27	0.15	Red
Trasncription			
LC19BE09	TATA box binding protein (TBP) associated factor (TAF)-like protein	8.16	6DPP
LC06CG11	nuclear protein ZAP	4.52	6DPP
LA19BC07	U2 snRNP auxiliary factor, large subunit	0.31	6DPP
FA24CA07	RNA helicas	0.26	6DPP
FB01DG04	zinc finger protein 1	36	30DPP
LB02CH01	protein RING zinc finger protein	3.73	30DPP
FB09BC08	multiprotein bridging factor 1	0.24	30DPP
LC19DG09	TAF5	0.13	30DPP
LC20DH10	splicing factor 3a protein	25.6	Orange
LA22DA02	spliceosome associated protein	12.6	Orange
LC06CG11	nuclear protein ZAP-like	5.6	Orange
FA36BG01	splicing factor Prp8	0.34	Orange
FA25CG07	zinc finger (CCCH-type) family protein	0.11	Orange
FA17DH01	bZIP transcription factor	0.11	Orange
Transport facilitation			
FB06CE11	myosin II heavy chain	0.27	6DPP
FA57CA09	sucrose transporter	0.07	6DPP
LA14DC08	kinesin light chain	0.27	30DPP
FA17DE07	sugar transporter	12	Orange
FA23BB04	aquaporin 1	8.09	Orange
FA09BH08	potassium channel regulatory factor	0.31	Orange
LA11DE12	myosin heavy chain	21.8	Red
FB17CA12	Nonspecific lipid-transfer protein A	2.52	Red

[a] Identification number in the NCBI, TIGR or TAIR EST databases. [b] Putative function and classification into functional categories according to BLAST search. [c] Mean ratio of the normalized signal intensity between the transformant (T) and the wild-type (WT) [d] Developmental stage of tomato fruit: 6 day-post-pollination (6DPP), 30 day-post-pollination (30DPP), coloring stage (Orange) and maturation stage (Red).

function or unannotated genes showed differing expression levels in the 30DPP, orange, and red stages, respectively.

Discussion

To better understand the contribution of HMGR to tomato fruit enlargement, a melon *HMGR* gene (*CmHMGR*) driven by the CaMV 35S promoter was introduced into tomato (Kobayashi et al. 2003). We selected the transgenic line CD-25 because it shows moderate expression of the transgene and sets larger fruit than the wild type. Maintenance of the line by self-pollination eventually led to a line that is homozygous for the transgene. The T_4 generation of the CD-25 line showed genetically stable expression of the *CmHMGR* mRNA and the corresponding protein as well as increased fruit size. Getting together with the previous reports (Higashi et al. 1999; Kato-Emori et al. 2001, Kobayashi et al. 2002; Kobayashi et al 2003), expression of HMGR protein, HMGR activity and cell proliferation were correlated during fruit development and resulted in increasing fruit size. Therefore, it is likely that the transgenic tomato fruit in this study has increased HMGR activity due to the increased expression of HMGR protein. Although it is still required HMGR enzyme assay, this transgenic line may be useful for understanding the roles of HMGRs in fruit development.

Length of the roots and hypocotyl was shorter in the *CmHMGR* overexpressing seedlings than in wild-type seedlings. In *Arabidopsis*, it has been reported that HMGR inhibitor-treated plants and *HMGR* loss-of-function mutants exhibited the same phenotype (Re et al. 1995; Kasahara et al. 2002; Suzuki et al. 2004; Rodríguez-Concepción et al. 2004). In these studies, blocking HMGR activity caused reduced cell expansion and cell division and altered morphology. Taken these together, our result suggests that HMGR activity is down-regulated in the seedling of *CmHMGR* overexpressing tomato. It has been reported that HMGR is involved in fruit enlargement in several plant species (Cowan et al. 1997; Kato-Emori et al. 2001; Kobayashi et al. 2002, Kobayashi et al. 2003). However, it is unknown how HMGR contributes to fruit enlargement. Therefore, we conducted a morphological analysis and molecular characterization of fruits. As expected, the T4 transgenic tomatoes steadily set fruits of 20-24% greater weight than those of the wild-type in both winter 2005 and spring 2006 (Table 1). Although the fruits showed increased length, diameter, and fresh weight, neither the fruit index nor the hundred-seed weight were significantly different. The lack of change in the hundred-seed weight may be due to the normal embryogenesis in the transgenic line. In view of the agricultural importance of tomato, the stable enlargement of fruit size may have an impact on fruit production.

The tomato genome contains at least four *HMGR* genes (Park et al. 1992). We examined the transcription levels of *SlHMGR1*, *SlHMGR2*, and *SlHMGR3* (Figure 3) but not that of *SlHMGR4*, since its sequence is not yet available in the database. In the transformant, all *SlHMGR*s showed suppressed mRNA levels throughout the developmental stages. There are two possibilities to explain this phenomenon. One is negative feedback regulation triggered by the compounds derived from MVA pathway. In animals and bacteria, negative feedback regulation of the MVA pathway by steroidal and non-steroidal compounds at the transcriptional and post-transcriptional levels has been observed (Dimster-Denk et al. 1995; Vallett et al. 1996; Mo et al. 2004). Another possibility is the co-suppression of endogenous *HMGR*s, as Jorgensen first reported in 1990. However, co-suppression of homologous genes generally reduces their mRNA to almost undetectable levels. Therefore, feedback regulation is more likely to occur in the fruit of this transgenic tomato line. In spite of the suppression of the tomato endogenous *HMGR*s, the transformants set significantly larger fruits (Table 3). As this fruit enlargement is consistent with earlier findings (Cowan et al. 1997; Kato-Emori et al. 2001), it is likely that the flow of the MVA pathway in this transgenic tomato is elevated.

The effects of *CmHMGR* overexpression on the sizes of fruit and seedling may imply that the roles of MVA pathway-derived compounds in growth regulation are diverse depending on the developmental stages and tissues. The MVA pathway supplies multiple products that are important for plant growth and development, including a great variety of isoprenoid products and phytohormones (Stermer et al. 1994). As alterations in the MVA pathway have an effect on plant growth regulators, the constitutive expression of *CmHMGR* may change the balance of these products in seedlings and fruits, bringing about the abnormal phenotypes.

A comprehensive analysis of the transcripts in tomato fruit was performed using a cDNA macroarray generated by JSOL. We first compared the expression of the genes involved in isoprenoid biosynthesis in the wild-type and the transformant (Table 2). The GPP synthase large subunit gene was significantly suppressed in the 30DPP and orange stages, but no obvious change was observed in the other genes in any of the stages. This supports the assumption that the reaction catalyzed by HMGR is the rate-limiting step of the MVA pathway. The unchanged expression of the genes related to the isoprenoid biosynthesis implies that those genes are not involved in the adjustment of MVA pathway, but *HMGR* moderates the impact of excessive *HMGR* expression, which is similar to that in mammalian system.

Subsequently, the expression levels of the other genes

on the macroarray were compared and differentially expressed genes were identified (Table 3). We initially focused on genes that may be related to cell division or cell expansion because such genes could directly affect fruit size. The expression level of a cDNA annotated as a plasma-membrane intrinsic protein (PIP)-type water channel protein aquaporin 1 gene was higher in orange-stage fruits of the transformant (Table 3). Aquaporins are channels that are important in the intake of water, and have also been suggested to be involved in cell division and expansion (Sugaya et al. 2001; O'Brien et al. 2002). These functions imply that aquaporin has an effect on fruit growth, but it is unclear whether aquaporins affect fruit size since the post-transcriptional regulation of aquaporin activity through phosphorylation has been reported (Chaumont et al. 2005).

In this study, we analyzed a tomato line that stably sets larger fruit, using a comprehensive analytical strategy. To obtain further knowledge, it will be necessary to conduct a fine analysis targeting a small number of genes that are selected based on these results.

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