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Transformation of Tomato with the Melon 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Leads to Increase of Fruit Size

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Abstract

The expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is involved in the determination of final fruit size of tomato (*Lycopersicon esculentum* Mill.). We developed transgenic tomatoes that over-expressed the melon (*Cucumis melo* L. *reticulatus*) *Cm-HMGR-CD* gene, which encodes the catalytic domain of HMGR. The derived transgenic plants expressed *Cm-HMGR-CD*. Flow cytometric analysis revealed that cell division and/or elongation in the fruit pericarp during early fruit development was induced more rapidly in the transgenic plant than in the wild-type control. The fruit size in transgenic plants was larger than that in controls. The growth of fruit pericarp also enhanced in transgenic plants. These results suggest that HMGR might stimulate pericarp development by activation of cell division and/or elongation (differentiation), and thereby affects fruit size. In addition, the constitutive expression of *Cm-HMGR-CD* affected plant morphology. Thus, transgenic plant growth was suppressed and the leaves accumulated higher levels of chlorophyll, as compared to wild-type control plants.

Key words: cell division, cell elongation, fruit development, 3-hydroxy-3-methylglutaryl coenzyme A reductase, tomato, transgenic plant.

Introduction

Fruit size regulation is a major issue in the development of higher plants. Fruit growth in higher plants takes place in two distinct stages: cell division and cell expansion (Gillaspy *et al.*, 1993). The earliest fruit growth is primarily due to active cell division, whereas enlargement to full size is due to cell expansion. Recent studies have suggested that the number of pericarp cells during the cell division stage determines the final fruit size (Bohner and Bangerth, 1988; Ho, 1996; Cowan *et al.*, 1997; Higashi *et al.*, 1999; Joubes *et al.*, 1999; Frary *et al.*, 2000). However, little is known about the regulatory mechanisms governing fruit size.

The expression and activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is required for early fruit development in tomatoes and avocados (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 the rate-limiting step of isoprenoid biosynthesis (Bach, 1986, 1987; Stermer *et al.*, 1994; Chappell, 1995a, b; McGarvey and Croteau, 1995; Weissenborn *et al.*, 1995). The isoprenoid compounds have diverse functions in a number of physiological responses, including synthesis of membrane sterols and plant growth regulators (cytokinin, abscisic acid, gibberellins, and brassinosteroids), electron transport, and production of sesquiterpenoid phytoalexins, which provides resistance to pathogens (Stermer *et al.*, 1994). High levels of HMGR expression and activity were 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). Therefore, HMGR might be induced during fruit development by demands for isoprenoid compounds for the cell

division and growth processes (Cowan *et al.*, 1997; Jelesko *et al.*, 1999). We previously isolated the melon *HMGR* gene (*Cm-HMGR*; GenBank accession number: AB021862). The expression of *Cm-HMGR* increased in the pericarp during the initial stages of cell division (Kato-Emori *et al.*, 2001; Kobayashi *et al.*, 2002). In addition, *Cm-HMGR* expression levels and HMGR activity were proportional to the mature melon size. These results strongly suggested that *HMGR* expression was closely associated with the determination of fruit size.

In this study, we developed transgenic tomato plants that over-expressed the melon *Cm-HMGR* gene, under the control of a cauliflower mosaic virus (CaMV) 35S promoter. Transgenic tobacco plants over-expressing the *HMGR* gene were used to elucidate the regulatory role of *HMGR* in the biosynthesis of isoprenoid compounds (Chappell *et al.*, 1995; Schaller *et al.*, 1995). However, the morphology and development of *HMGR*-expressing plants has not been fully analyzed. For transformation, we used tomato that is an ideal model for investigating fruit development (Gillaspy *et al.*, 1993). We subsequently analyzed the fruit development of transgenic tomato plants.

Materials and Methods

Construction of the Cm-HMGR-CD expression vector

Since the catalytic domain of HMGR is sufficient for enzymatic activity (Schaller *et al.*, 1995), we used the carboxyl terminal region of Cm-HMGR, designated Cm-HMGR-CD, for transformation. In order to produce the *Cm-HMGR-CD* expression construct, we used a sense primer: 5'-GGAAGCTTTCTAGA*ATG*AACTCGAGCTAT GCTGCACCTCGTTCC-3', and an antisense primer: 5'-TCCTCTGCAGTGCCTCAC GG-3'. A translation start codon (italics), and the *Hin*dIII, *Xba*I and *Pst*I restriction sites (underlined) are shown. PCR amplification was carried out using a full length *Cm-HMGR* cDNA (pBS-HM; Kato-Emori *et al.*, 2001) as the template. The PCR-amplified fragment was digested with *Hin*dIII and *Pst*I and replaced with a *Hin*dIII-*Pst*I fragment of pBS-HM comprising the 5'-region of the *Cm-HMGR* cDNA. The resulting *Cm-HMGR-CD* gene encoded residues 134-588 of Cm-HMGR. The *Xba*I-*Sac*I fragment carrying *Cm-HMGR-CD* was then cloned into pBI121 (Clontech Laboratories, Palo Alto, CA) which was subsequently transferred into *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method (Ann *et al.*, 1988).

Transformation and regeneration of tomato plants

The transformation and regeneration of tomato plants (*Lycopersicon esculentum* Mill. cv. Moneymaker, kindly provided by Dr. M. Matsui, RIKEN, Japan) were carried out according to the method of Ohyama *et al.* (1995). The primary transformants were selected in the presence of 100 μg ml^{-1} kanamycin and screened for the presence of the *Cm-HMGR-CD* sequence by PCR. Three independent transformants were obtained and propagated *in vitro*. The transformants were planted out and grown in a containment greenhouse for fruit growth analysis. The primary transformants were self-crossed and T_1 seedlings were screened, as described above.

Protein preparation, SDS-PAGE and Western blot analysis

The detailed methods for protein preparation, SDS-PAGE, and Western blotting have been described previously (Kobayashi *et al.*, 2002). Soluble protein fractions, prepared from leaves and fruits by centrifugation at 100,000*g*, were subjected to Western blot analysis. Tomato HMGR proteins containing transmembrane domains co-sedimented with microsomal membranes during centrifugation (Enjuto *et al.*, 1994; Campos and Boronat, 1995; Lumbreras *et al.*, 1995; Denbow *et al.*, 1996), while the Cm-HMGR-CD protein lacking the putative transmembrane domains was located in the soluble protein fraction. An anti-Cm-HMGR-CD antibody (Kobayashi *et al.*, 2002) and an anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad, Hercules, CA) were used at 1:1000 and 1:3000 dilutions, respectively.

Morphological analysis and the development of Cm-HMGR-CD-expressing tomatoes

Transgenic plants and wild-type controls (cv. Moneymaker) were grown in a containment greenhouse. There were fewer flowers on transgenic plants than on wild-type plants. Thus, we adjusted the number of fruits in the control to equal that in transgenic plants. The excess fruits in both transgenic and wild-type plants were removed after fruit set (DPA 6), while taking plant size (i.e., the number of leaves) into account. The number of fruits was adjusted to approximately five compound leaves per fruit. The periods of flower senescence, fruit enlargement and fruit ripening were monitored. The fruit size was also measured on each day post anthesis (DPA).

Flow Cytometric Analysis

Flow cytometry was performed as described

previously (Kobayashi *et al.*, 2002). Nuclei prepared from tomato fruit pericarps were stained with 4,6-diamidino-2-phenylindole, and subjected to flow cytometric analysis.

Chlorophyll quantification

The extraction and spectrophotometric quantification of chlorophyll were performed as described (Arnon, 1949). Approximately 0.5 g of leaves was extracted three times with 5 ml of 80% (v/v) acetone containing 2.5% (w/v) $Mg(CO_3)_2$, and the resultant extracts were subjected to spectrophotometry. The total concentration of chlorophyll was calculated as follows: 20.2 x A_{645} + 8.02 x A_{663} (mg ml^{-1}). The results are presented as the total chlorophyll content per gram of fresh leaf. The chlorophyll a/b ratio was calculated according to the formula: 12.7 x A_{663} + 2.69 x A_{645} / 22.9 x A_{645} − 4.68 x A_{663}.

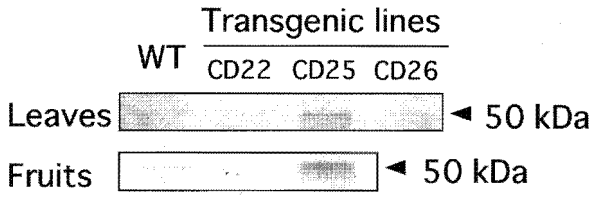

Fig. 1 Immunological detection of Cm-HMGR-CD in T_0 transgenic plants. Five μg of soluble proteins, prepared from leaves and fruit pericarps (10 DPA) of transgenic plants, and a wild-type control (WT), were subjected to Western blot analysis with anti-Cm-HMGR-CD antibody.

Results

Cm-HMGR-CD expression in T_0 transgenic plants

Three lines of transgenic T_0 plants, CD-22, CD-25 and CD-26, were generated by *Agrobacterium*-mediated transformation. Lines CD-22 and CD-25 set fruits while line CD-26 did not set any fruits.

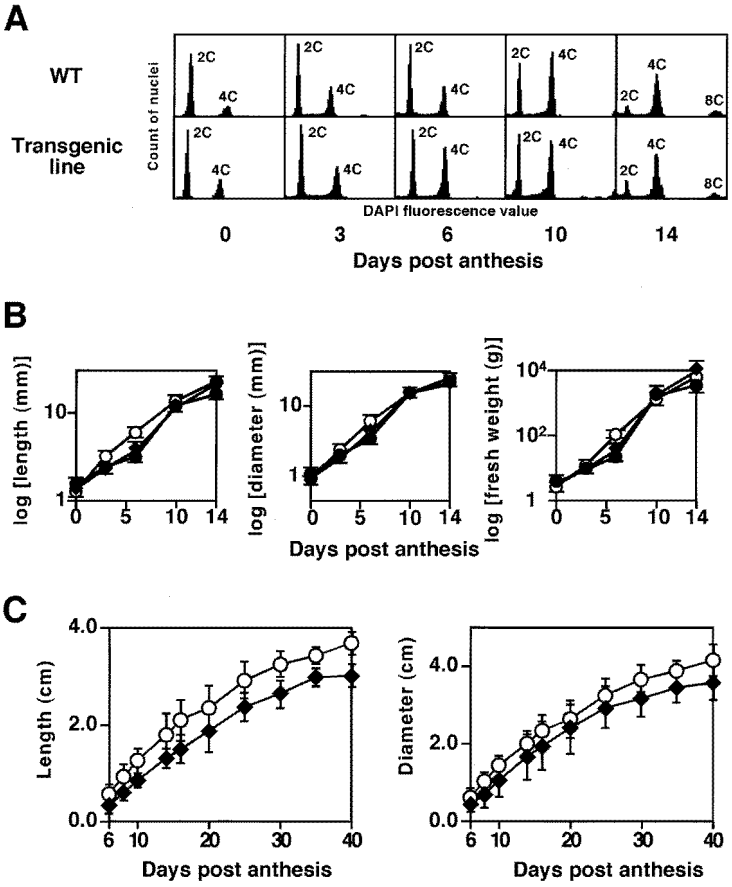

Fig. 2 Fruit development in transgenic plants expressing *Cm-HMGR-CD*. (A) Flow cytometric analysis of the fruit pericarp. Histograms show two peaks, which represent G1- and G2- phase cells with 2C and 4C nuclear DNA levels respectively. WT, wild-type control. (B) Fruit growth during early fruit development. The data shown represent the average values for at least five distinct measurements of fruit length, diameter and fresh weight with standard deviations. Open circles, transgenic line CD25; closed circles and diamonds, wild-type controls. (C) Fruit enlargement in an individual (CD25-3) derived from the transgenic line CD25. The data shown represent the mean values of fruit length and diameter for six distinct measurements with standard deviations. Open circles, transgenic line CD25; closed diamonds, wild-type controls.

Western blotting revealed accumulations of Cm-HMGR-CD protein in transgenic T_0 plants (**Fig. 1**). Cm-HMGR-CD was detected in the soluble protein fraction as a 50-kDa band, which closely matches the size of Cm-HMGR-CD (51.6 kDa), predicted from the DNA sequence. Interestingly, one transgenic line, CD25, showed elevated levels of Cm-HMGR-CD in leaves, as compared to the wild-type control. Expression of Cm-HMGR-CD was also detected in fruits of CD25. Therefore, CD25 was used for further analysis.

Morphology and fruit development in T_0 transgenic plants expressing Cm-HMGR-CD

Flow cytometric analysis of pericarp cells revealed active cell division and/or elongation (differentiation) in the T_0 transgenic line CD25 during early fruit development (**Fig. 2A**). The 4C peak of CD25 on DPA 6 was two-fold higher than that of the wild-type control. The higher peak of 4C cells indicates that the proportion of dividing cells in the pericarp might be increased by activation of cell proliferation. Another possibility is that cell differentiation might have progressed earlier than the wild-type control and, as a consequence, ploidy level was elevated. Therefore, our result suggests that cell division and/or elongation in the pericarp of CD25 was rapidly activated, as compared to that of the wild type. The occurrence of endopolyploidity by DPA 14 indicated cell division cessation. However, there were no differences in peak patterns between CD25 and the wild type after DPA

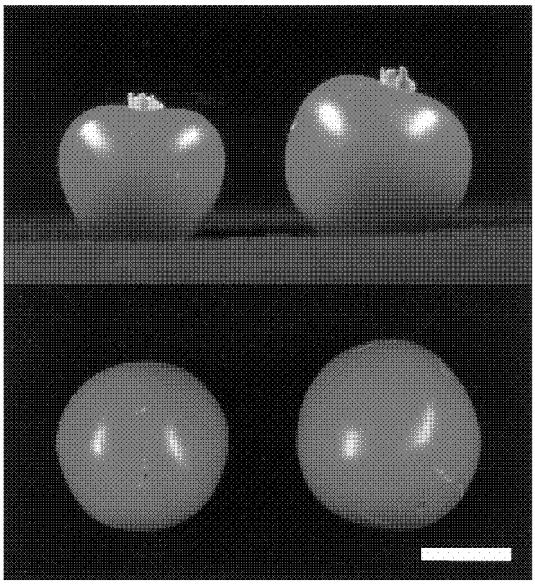

Fig. 3 Morphology of fruits of T_0 transgenic plants over-expressing Cm-HMGR-CD. Fruit of wild-type control (left) and T_0 transgenic line CD25 (right). Bar = 2 cm.

Table 1 Fruit size in T_0 transgenic plants expressing *Cm-HMGR-CD*

Line	No. of clones	Number of fruits	Length (cm)[1)]	Diameter (cm)[1)]	Fresh weight (g)[1)]	Pericarp thickness (mm)[1)]
Control	1	6	3.4 ± 0.3a	4.2 ± 0.3a	42.3 ± 6.6a	4.4 ± 0.6a
	2	7	3.6 ± 0.1a	4.2 ± 0.2ab	42.4 ± 5.0a	4.7 ± 0.5a
	3	6	3.6 ± 0.2a	4.2 ± 0.4a	39.2 ± 8.4a	4.8 ± 0.8a
CD25	1	4	4.3 ± 0.1b	4.7 ± 0.4c	53.2 ± 6.9b	7.0 ± 0.0b
	2	6	4.2 ± 0.2b	4.5 ± 0.3bc	46.7 ± 6.3ab	6.3 ± 0.8b
	3	6	4.2 ± 0.2b	4.7 ± 0.4c	55.2 ± 11.3b	6.4 ± 1.1b

[1)] Mean values with standard deviations. Statistically significant differences ($P< 0.05$), calculated according to the Student's t-test, are indicated by different characters.

Table 2 Fruit development in T_0 transgenic plants expressing *Cm-HMGR-CD*

Line	No. of clones	Number of flowers	Number of seeds per fruit	Developmental stage (days)		
				Flower senescence[1)]	Fruit growth[2)]	Fruit ripening[3)]
Control	1	40	103.3	2.5	53 ± 7	5 ± 2
	2	41	86.8	2.7	48 ± 4	5 ± 1
	3	61	88.2	2.8	56 ± 6	6 ± 1
CD25	1	10	90.0	2.3	52 ± 5	6 ± 1
	2	29	43.3	2.7	48 ± 2	5 ± 1
	3	24	71.3	2.4	46 ± 1	4 ± 1

[1)] Days between full blooming and flower senescence.

[2)] Days between flowering and breaker stages.

[3)] Days between breaker and red-ripe stages.

10. The active cell division and/or elongation in the CD25 pericarp was accompanied by only a slight stimulation of fruit growth on DPA 6 (**Fig. 2B**). The CD25 fruit grew more rapidly than that of the wild type between DPA 10 and 30 (**Fig. 2C**), and at maturation the CD25 fruit was larger than that of the control (**Fig. 3**). The length, diameter, and fresh weight of CD25 fruits were 120%, 110%, and 130% those of control fruits, respectively (**Table 1**). The pericarp thickness in CD25 fruits increased to 140% that of control fruits. On the other hand, the number of flowers in CD25 plants was one-third that of the control. Nevertheless, other parameters of development, such as flower senescence, fruit enlargement and ripening, were similar for CD25 and control plants (**Table 2**). The number of seeds per fruit was not statistically different between the wild-type control and transgenic line CD25, except for one CD25 plant in which the number of seeds decreased dramatically.

The leaves of CD25 transgenic plants were dark green in color and were smaller than the wild-type leaves. The total chlorophyll content of CD25 plants was 1.5-fold higher than in the control, whereas the chlorophyll a/b ratio was similar in CD25 and the control (**Table 3**).

Table 3 Leaf chlorophyll content in T_0 transgenic plants expressing *Cm-HMGR-CD*

Line	No. of clones	Total chlorophyll [mg Chl (g FW)$^{-1}$][1)]	Chl a / Chl b
Control	1	1.6 ± 0.01	2.5
	2	1.2 ± 0.01	3.7
	3	1.0 ± 0.02	3.5
CD25	1	2.0 ± 0.00	3.2
	2	3.0 ± 0.01	2.8
	3	2.4 ± 0.01	2.7

[1)] Mean values (n=4) with standard deviations. The differences between the control and CD25 lines were statistically significant according to the Student's *t*-test ($P < 0.01$).

Fruit development in T_1 progeny of Cm-HMGR-CD-expressing plants

Five independent T_1 progenies of the transgenic CD25 line were divided into two groups according to their *Cm-HMGR-CD* expression levels (**Fig. 4**). Two T_1 lines, CD25-1 and CD25-3, showed low levels of Cm-HMGR-CD, whereas the other three lines, CD25-2, CD25-4 and CD25-5, showed remarkably high production of Cm-HMGR-CD. These two groups differed in fruit size and plant development. In CD25-1 and CD25-3 plants, fruit enlargement was elevated (**Table 4**), but the plant development was slightly suppressed, as compared to wild-type controls. In contrast, the growth of CD25-2, CD25-4 and CD25-5 was strongly suppressed. In addition, these T_1 plants had fewer and

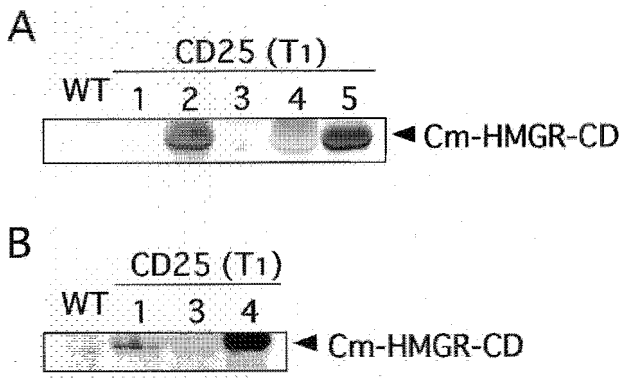

Fig. 4 Accumulation of Cm-HMGR-CD protein in T_1 transgenic plants. Soluble proteins were prepared from leaves of five T_1 progenies of the transgenic line CD25. Protein extracts, in aliquots of 5 μg (A) or 20 μg (B), were subjected to Western blot analysis with anti-Cm-HMGR-CD antibody.

Table 4 Fruit size in T_1 progeny of *Cm-HMGR-CD*-expressing plants

Line[1)]	Number of fruits	Number of leaves per fruit	Length[2)] (cm)	Diameter[2)] (cm)	Fresh weight[2)] (g)	Pericarp thickness[2)] (mm)
Control-1	8	5.1	3.7 ± 0.2a	4.4 ± 0.3a	38.4 ± 6.6 a	4.5 ± 0.9a
Control-2	8	4.7	3.7 ± 0.2a	4.2 ± 0.2b	35.5 ± 5.0a	5.0 ± 0.5a
CD25-1	12	4.4	4.1 ± 0.2b	4.4 ± 0.3ac	42.3 ± 6.9ab	6.3 ± 1.0b
CD25-3	6	5.4	4.1 ± 0.2b	4.6 ± 0.3c	46.5 ± 6.3b	6.7 ± 0.5b
CD25-4	1	21	2.9	3.0	13.2	2.0
CD25-5	1	15	2.9	2.7	12.0	3.0

[1)] CD-25-2 had no fruits.

[2)] Mean values with standard deviations. The different characters indicate statistically significant differences ($P< 0.05$), calculated using the Student's *t*-test. We did not perform statistical analysis in CD25-4 and CD25-5, because of insufficient number of fruits.

much smaller fruits than the control plants (**Table 4**). CD25-2 had no flowers and fruits.

Discussion

Using a reverse genetic approach, we have demonstrated that over-expression of *HMGR* gene leads to an increase in fruit size (**Fig. 3**). The pericarp thickness of the *Cm-HMGR-CD*-expressing tomatoes increased as compared to that of the wild type fruits (**Table 1, 4**). The pericarp cell number defined during the cell division stage is a major determinant of fruit size (Bohner and Bangerth, 1988; Cowan *et al.*, 1997; Higashi *et al.*, 1999). The division of pericarp cells results in an increase of the cell layers in the fruit pericarp. Because the fruit pericarp is one of the main structures that directly affect final size of fleshy fruit (Atta-Aly *et al.*, 1999; Frary *et al.* 2000), the thickening of the fruit pericarp induces the enlargement of fruit size. Therefore, such stimulation of pericarp growth in Cm-HMGR-CD-expressing fruits might lead to the increase in final fruit size. These results suggest that *HMGR* expression is involved in fruit enlargement.

Recent studies suggest that HMGR has essential role in cell division and growth in fruit pericarp during early fruit development (Narita and Gruissem, 1989; Enjuto *et al.*, 1995a, b). HMGR levels in melon and avocado fruits have been correlated with progression of cell division in the pericarp (Cowan *et al.*, 1997; Kato-Emori *et al.*, 2001). Jelesko *et al.* (1999) revealed that tomato *HMGR* expression is induced during early fruit development to ensure a continuous supply of the various isoprenoid compounds that are crucial to the cell division process. Therefore, over-expression of *Cm-HMGR-CD* might increase the pool of mevalonic acid, which would allow rapid promotion of cell division and growth during early fruit development (**Fig. 2A**). However, transgenic plants seemingly showed slightly altered fruit growth patterns during the early stage of fruit development (**Fig. 2B, C**). This might be due to different growth pattern between the cell division and expansion stages (Gillaspy *et al.*, 1993; Atta-Aly *et al.*, 1999). Although the final fruit size is dependent on the cell number in the pericarp (defined during the cell division stage), fruit enlargement is a product of pericarp cell expansion after the cell division stage.

Constitutive expression of *HMGR* influenced not only the fruit size regulation but also plant vegetative growth. Transgenic tomato plants over-expressing *Cm-HMGR-CD* had an increased level of the total chlorophyll content of leaves, but did not affect the chlorophyll a and b compositions (**Table 3**). On the other hand, *Cm-HMGR-CD*-expressing tomatoes showed growth suppression, i.e., they had fewer flowers and smaller leaves than wild-type control plants (**Table 2**). In particular, we found that high amounts of Cm-HMGR-CD in transgenic T_1 lines CD25-2, CD25-4 and CD25-5 strongly inhibited their plant growth. Although the reasons for these severe effects on plant development are unclear, high level *Cm-HMGR-CD* expression in whole plant probably brings about an excessive production of isoprenoid compounds, which in turn adversely affects diverse physiological responses and disturbs carbon metabolism (Stermer *et al.*, 1994; McGarvey and Croteau, 1995; Weissenborn *et al.*, 1995; Chappell, 1995, 1996). It will be necessary to perform physiological and biochemical analyses of vegetative growth in Cm-HMGR-CD-expressing plants in detail.

In this study, transgenic plants expressing *Cm-HMGR-CD* represent a powerful application of *HMGR* gene manipulation to fruit size development. However, it is worth noting that the constitutive expression of *Cm-HMGR-CD* was accompanied by plant growth suppression. Therefore, a moderate level of *Cm-HMGR-CD* expression appears to be sufficient for the stimulation of tomato fruit enlargement. Further studies are required on the *Cm-HMGR*-promoter regulating fruit-specific expression for better understanding of fruit development and efficient gene manipulation of fruit size regulation.

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