

RESEARCH PAPER

Complete blockage of the mevalonate pathway results in male gametophyte lethality

Masashi Suzuki¹, Shoko Nakagawa^{1,2}, Yukiko Kamide¹, Keiko Kobayashi^{1,*}, Kiyoshi Ohyama¹, Hiromi Hashinokuchi¹, Reiko Kiuchi¹, Kazuki Saito^{1,3}, Toshiya Muranaka^{1,4,†} and Noriko Nagata²

¹ RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045, Japan

² Graduate School of Science, Japan Women's University, 2-8-1, Mejirodai, Bunkyo-ku, Tokyo, 112-8681 Japan

³ Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba, Chiba, 263-8522 Japan

⁴ Kihara Institute for Biological Research, Yokohama City University, 641-12, Maioka-cho, Totsuka-ku, Yokohama, Kanagawa, 244-0813 Japan

Received 22 December 2008; Revised 23 February 2009; Accepted 23 February 2009

Abstract

Plants have two isoprenoid biosynthetic pathways: the cytosolic mevalonate (MVA) pathway and the plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Since the discovery of the MEP pathway, possible metabolic cross-talk between these pathways has prompted intense research. Although many studies have shown the existence of such cross-talk using feeding experiments, it remains to be determined if native cross-talk, rather than exogenously applied metabolites, can compensate for complete blockage of the MVA pathway. Previously, *Arabidopsis* mutants for *HMG1* and *HMG2* encoding HMG-CoA reductase (HMGR) were isolated. Although it was shown that HMGR1 is a functional HMGR, the enzyme activity of HMGR2 has not been confirmed. It is demonstrated here that *HMG2* encodes a functional reductase with similar activity to HMGR1, using enzyme assays and complementation experiments. To estimate the contribution of native cross-talk, an attempt was made to block the MVA pathway by making double mutants lacking both *HMG1* and *HMG2*, but no double homozygotes were detected in the progeny of self-pollinated *HMG1/hmg1 hmg2/hmg2* plants. *hmg1 hmg2* male gametophytes appeared to be lethal based on crossing experiments, and microscopy indicated that ~50% of the microspores from the *HMG1/hmg1 hmg2/hmg2* plant appeared shrunken and exhibited poorly defined endoplasmic reticulum membranes. *In situ* hybridization showed that *HMG1* transcripts were expressed in both the tapetum and microspores, while *HMG2* mRNA appeared only in microspores. It is concluded that native cross-talk from the plastid cannot compensate for complete blockage of the MVA pathway, at least during male gametophyte development, because either *HMG1* or *HMG2* is required for male gametophyte development.

Key words: Anther, cross-talk, HMG-CoA reductase, isoprenoid, male gametophyte, MEP pathway, MVA pathway, pollen, sterol, tapetum.

Introduction

Plants produce a wide variety of isoprenoids by the condensation of common five-carbon intermediates, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Over the course of evolution, plants have maintained the well-known eukaryotic mevalonate (MVA) pathway for cytosolic synthesis of IPP and DMAPP,

and this pathway was considered to be the sole synthetic source of these compounds for several decades. However, plants have recently been discovered also to make use of the prokaryotic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway from the endosymbiotic ancestral cyanobacteria in plastids (Rohdich *et al.*, 2001; Fig. 1). Under normal

* Present address: Faculty of Agriculture, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka, 812-8581 Japan.

† To whom correspondence should be addressed. E-mail: muranaka@yokohama-cu.ac.jp

© 2009 The Author(s).

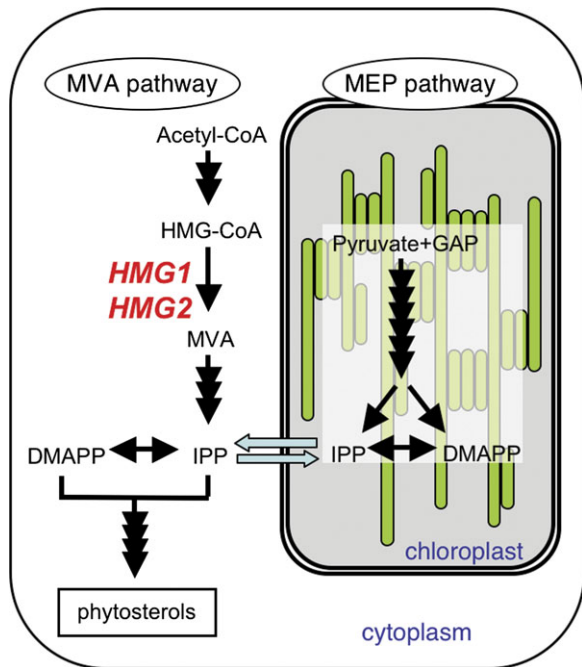


Fig. 1. Outline of the two isoprenoid biosynthetic pathways in an *Arabidopsis* cell: the MVA and MEP pathways. Blue arrows indicate metabolic flow between the cytosol and plastid. Two genes, *HMG1* and *HMG2*, encoding HMGR are shown in red letters. MVA, mevalonate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GAP, glyceraldehyde 3-phosphate.

physiological conditions, the two pathways are compartmentalized. Cytosolic isoprenoids (e.g. sterols and brassinosteroids) and mitochondrial isoprenoids (e.g. side chains of ubiquinone) are synthesized via the MVA pathway, while the MEP pathway is used to synthesize plastidic isoprenoids (e.g. carotenoids and the side chains of chlorophylls and plastoquinones) and some isoprenoid-type phytohormones (e.g. gibberellins, abscisic acid, and cytokinins).

Cytosolic IPP and DMAPP are synthesized in seven enzymatic steps from acetyl-CoA via the MVA pathway, and the HMG-CoA reductase (HMGR) reaction is considered to be rate-limiting in this pathway (Schaller *et al.*, 1995; Manzano *et al.*, 2004; Suzuki *et al.*, 2004). The biological importance of HMGR is further emphasized by the existence of highly specific natural inhibitors of the enzyme including mevinolin (Alberts *et al.*, 1980), also known as lovastatin, and its analogues from various rhizopheric ascomycetes (Bach *et al.*, 1990), and their use as growth inhibitors and antibiotics. Earlier work revealed that mevinolin was an efficient plant growth regulator primarily through its inhibition of *de novo* phytosterol biosynthesis (Bach and Lichtenthaler, 1987; Bach *et al.*, 1990). Mevinolin and its analogues blocked cell proliferation in tobacco (Bach and Lichtenthaler, 1987; Hata *et al.*, 1987; Crowell and Salaz, 1992; Hemmerlin and Bach, 1998; Randall *et al.*, 1993; Morehead *et al.*, 1995), *Helianthus tuberosus* (Ceccarelli and Lorenzi, 1984), *Medicago sativa* (Hata *et al.*, 1987), and *Acer pseudoplatanus* (Ryder and Goad, 1980). Plant HMGRs are encoded by a small family

of genes that are differentially expressed upon various internal and external stimuli, and the transcriptional and post-transcriptional regulation of HMGRs have been investigated (Dale *et al.*, 1995; Learned, 1996; Newman and Chappell, 1997). Genetic approaches have demonstrated some of the novel HMGR regulatory mechanisms in plants relative to animals (Rodríguez-Concepción *et al.*, 2004; Kobayashi *et al.*, 2007).

Feeding experiments in plants have indicated the occurrence of cross-talk between the MVA and MEP pathways. Labeled mevalonolactone (MVL) and 1-deoxy-D-xylulose (DX) are incorporated into plastidic and cytosolic isoprenoids, respectively, in *Lemna gibba* (Schwender *et al.*, 2001), *Arabidopsis* (Kasahara *et al.*, 2002), and tobacco Bright Yellow-2 (BY-2) cells (Hemmerlin *et al.*, 2003). Exogenously applied MVL partially rescues developmental arrest in *cla1-1*, an *Arabidopsis* mutant with a disrupted MEP pathway (Nagata *et al.*, 2002), and partially rescues growth inhibition of BY-2 cells treated with fosmidomycin, a DX reductoisomerase inhibitor (Hemmerlin *et al.*, 2003). Exogenously applied DX partially rescues the growth inhibition in *Arabidopsis* plants (Kasahara *et al.*, 2002) and BY-2 cells (Hemmerlin *et al.*, 2003) treated with mevinolin. However, these feeding studies using exogenously applied metabolites were insufficient to determine whether mutual and native cross-talk can compensate for complete blockage of one of the isoprenoid biosynthetic pathways.

Mutants lacking one functional isoprenoid biosynthetic pathway are very useful to examine native cross-talk. The identification in *Arabidopsis* of the isoprenoid biosynthesis mutant *cla1-1*, a null mutant for a DX synthase (*DXS*) gene, is one important example (Mandel *et al.*, 1996; Araki *et al.*, 2000; Estévez *et al.*, 2000). Despite the albino phenotype of *cla1-1*, two *DXS* homologous genes exist in the *Arabidopsis* genome in addition to *CLA1* (Lange and Ghassemian, 2003). It remains unclear whether a *cla1* mutation is sufficient for complete shutdown of the MEP pathway. Some MEP pathway mutants have been reported, including *ispF-1* for 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS; Hsieh and Goodman, 2006), *clb4* for hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (HDS; Gutiérrez-Nava *et al.*, 2004), and *clb6* for 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase (HDR; Guevara-García *et al.*, 2005; Hsieh and Goodman, 2005). These three enzymes are encoded by a single gene in *Arabidopsis*. When grown on sucrose-supplemented medium, even homozygous mutants completely lacking the MEP pathway are viable and capable of germinating.

The seven enzymatic reactions from acetyl-CoA to IPP and DMAPP are encoded by 12 genes in *Arabidopsis* (Suzuki and Muranaka, 2007). Among these 12 genes, *HMG1* and *HMG2*, encoding HMGR (Suzuki *et al.*, 2004), as well as *IPI1* and *IPI2* (also referred to as *ID11* and *ID12*), encoding IPP isomerase (Okada *et al.*, 2008; Phillips *et al.*, 2008; Sapir-Mir *et al.*, 2008) have been analysed genetically. Whereas *IPI1* has been found in multiple subcellular locations: the cytosol (Okada *et al.*, 2008), the plastid (Phillips *et al.*, 2008) or the peroxisome (Sapir-Mir *et al.*, 2008), *IPI2*

appears in the three cases to be mainly localized in the mitochondria. Thus, it is still not well understood which IPI isoform is devoted to the MVA pathway. On the contrary, HMGR is a key enzyme of the MVA pathway. The isolation and characterization of *hmg1* and *hmg2* mutants for the two HMGR genes in *Arabidopsis* have previously been reported (Suzuki *et al.*, 2004). Although *hmg1* mutants show pleiotropic phenotypes (dwarfism, early senescence, and male sterility), *hmg2* mutants exhibit no morphological phenotypes (Suzuki *et al.*, 2004). HMGR1 enzyme activity has been identified using yeast complementation (Learned and Fink, 1989) and *in vitro* analysis using the catalytic domain of HMGR1 expressed in *Escherichia coli* (Dale *et al.*, 1995). The function of HMGR2 is thought to be almost equal to that of HMGR1 based on the findings that *hmg2* has slightly higher sensitivity to lovastatin than do wild-type (WT) plants (Suzuki *et al.*, 2004) and that the sterol and triterpenoid levels of *hmg2* are slightly lower than those of WT plants (Ohyama *et al.*, 2007); however, direct evidence that *HMG2* encodes a functional HMGR has not yet been presented. To examine the influence of complete blockage of the MVA pathway on the plant phenotype, the generation and characterization of an *hmg1 hmg2* double mutant is important. Prior to attempting to generate the double mutant, the biochemical and physiological functions of HMGR2 were examined by an enzyme assay and *hmg1* complementation test using *HMG2*. It was found that the *hmg1 hmg2* double mutant is lethal during male gametophyte development.

Materials and methods

Plant growth conditions

Arabidopsis thaliana plants (ecotype Wassilewskija (WS), ecotype Columbia (Col), *hmg1-1* (WS background; Suzuki *et al.*, 2004), *hmg1-2* (WS background; Suzuki *et al.*, 2004), *hmg2-1* (Col background; Suzuki *et al.*, 2004), and *qrt1-1* (Landsberg *erecta* background; Preuss *et al.*, 1994) were germinated on 1× Murashige-Skoog (MS) plates containing 3% sucrose and 0.8% agar. For the HMGR enzyme assay and sterol analysis, 1-week-old seedlings were transplanted into 1× MS liquid medium containing 3% sucrose and cultured for one additional week. For RNA extraction, seedlings were grown on MS plates for 2 weeks.

HMGR enzyme assay and sterol analysis

The preparation of the microsomal fraction was performed as described by Kobayashi *et al.* (2007). HMGR activity was measured as described by Chappell *et al.* (1995). Sterols were extracted from freeze-dried plant tissues (2-week-old seedlings; 10 mg) and quantified as described by Suzuki *et al.* (2004).

RT-PCR analysis

RT-PCR analysis of *XTR9* and *extensin-like protein* was performed as described by Suzuki *et al.* (2004). The relative

quantification of *HMGI* and *HMG2* was performed using an Applied Biosystems 7500 Real Time PCR System, using the following primers and probes: *HMG1*-fwd, 5'-GGCGT-GACAAGATCCGTTACA-3'; *HMG1*-rev, 5'-GCAATA-ATGGCGCCGAGTT-3'; *HMG1*-probe, 5'-CACGTCGT-CACTATCACA-3'; *HMG2*-fwd, 5'-TACTCGGTGT-GAAAGGATCAAACA-3'; *HMG2*-rev, 5'-CCGAACCA-GCCACTATTCTTG-3'; *HMG2*-probe, 5'-AGAAACCT-GGCTCGAACGCACAGC-3'; 18SrRNA-fwd, 5'-CGGC-TACCACATCCAAGGAA-3'; 18SrRNA-rev, 5'-GCTGGA-ATTACCGCGGCT-3' and 18SrRNA probe, 5'-TGCTG-GCACCAGACTTGCCCTC-3'.

Construction of chimeric genes with *HMG1* promoter::*HMG1S* and *HMG1* promoter::*HMG2* and transformation of *Arabidopsis*

The *HMG1* promoter (Suzuki *et al.*, 2004) was connected to *HMG1S* or *HMG2* cDNA using PCR. These gene cassettes were digested with *EcoRI* and *XhoI* and cloned into these sites in pENTR™1A (Invitrogen, Carlsbad, CA, USA). The resultant entry clones were used for the *attL*×*attR* (LR) recombination reaction between GATEWAY™-converted binary vectors pBCR112 (H Seki *et al.* unpublished data). The transformation of *Arabidopsis* was performed as described previously (Suzuki *et al.*, 2004).

Light microscopy

For anther observations, flower buds were selected just before flowering. The anthers were fixed in 2% glutaraldehyde, and a coverslip was placed over the sample and pressed onto the slide. For pollen grain observations, flowers were selected just after flowering. The pollen grains were spread on a glass slide and fixed in 2% glutaraldehyde, and then stained with 2 µg ml⁻¹ DAPI. The samples were observed using an I× 70 fluorescence microscope (Olympus, Tokyo, Japan).

Electron microscopy

Several days before flowering, the anthers of flower buds were fixed for 20 h in 4% glutaraldehyde and 4% paraformaldehyde and then post-fixed with 2% osmium tetroxide for 20 h. The fixed samples were dehydrated through an ethanol series and embedded in Spurr's resin. Ultra-thin sections (70 nm) were double-stained with uranyl acetate and lead citrate, and then observed using a JEM-1200 EX transmission electron microscope (Jeol, Tokyo, Japan).

In situ hybridization

Arabidopsis (ecotype WS) flower buds were fixed with paraformaldehyde, dehydrated through a conventional ethanol series, and embedded in paraffin. Sections (5 µm) were made with a microtome (RM2135; Leica, Vienna, Austria) and deparaffinized with xylene. The sections were probed with sense or antisense RNA probes. For the *HMG1* probe, a 356 bp *HMG1* cDNA 3' untranslated region (UTR)

fragment was amplified using the primers 5'-CATGATCT-GAATCTGAATCATCATCCT-3' and 5'-CCAAACG-CAACTGCACCATAACA-3'. For the *HMG2* probe, a 420 bp *HMG2* cDNA 3' UTR fragment was amplified using the primers 5'-AGACATTGGCCCTTCGTCTCAAGTC-3' and 5'-ACCATATCACTCCTCCATCGTCTGC-3'. The amplified fragments were cloned into pCR-II TOPO vectors (Invitrogen; Carlsbad, CA, USA). Digoxigenin (DIG)-labelled *HMGI* and *HMG2* sense and antisense RNA probes were synthesized using a DIG RNA labelling kit (Roche, Basel, Switzerland). After hybridization and washing, the sections were incubated in a 1:2000 dilution of anti-DIG antibody coupled with alkaline phosphatase. The colour was developed using a detection buffer (100 mM TRIS-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, and 0.1% Tween-20) containing 18 µg ml⁻¹ of 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) solution (Roche).

Results

HMG2 encodes a functional HMGR

To examine the activity of HMGR1 and HMGR2 in plants, HMGR assays were performed using microsomal fractions prepared from 2-week-old seedlings of WS, Col, *hmg1-1*, and *hmg2-1*. The HMGR activity of *hmg1-1* was approximately 20% that of the WT (151 ± 6.9 pmol min⁻¹ mg⁻¹ protein), whereas the HMGR activity of *hmg2-1* was comparable to that of the WT (Fig. 2A). The remaining 20% activity in *hmg1-1* should be derived from HMGR2. This result biochemically indicates that HMGR2 has HMGR activity, as does HMGR1.

The cellular function of HMGR2 was examined. Because *hmg1* shows pleiotropic phenotypes such as dwarfism, early senescence, and male sterility (Suzuki *et al.*, 2004), it was determined whether *HMG2* could complement *hmg1*. Transgenic *hmg1-1* plants expressing *HMG2* cDNA driven by an HMGI promoter were generated and characterized. Transgenic *hmg1-1* plants expressing *HMG1S* cDNA driven by an HMGI promoter were used as positive controls. Two kinds of transcript encoded by *HMGI*, i.e. *HMG1S* and *HMG1L*, occur in *Arabidopsis* (Lumbreras *et al.*, 1995). Because *HMG1S* is the major transcript of *HMGI*, *HMG1S* cDNA was used as a positive control for the complementation test with *hmg1-1*. Several independent lines of these transgenic plants were generated in an *hmg1-1* homozygous background. Segregation analysis of the kanamycin resistance trait indicated that these lines contained a single T-DNA insertion and were homozygous with respect to the transgene. Among these lines, lines 1.1 and 1.4 for HMGI promoter::*HMG1S* and lines 2.13 and 2.23 for HMGI promoter::*HMG2* were selected for further characterization. The expression of *HMGI* and *HMG2* in each line was analysed using quantitative RT-PCR. The expression of *HMGI* in line 1.1 was much higher than that of WT plants, and *HMGI*

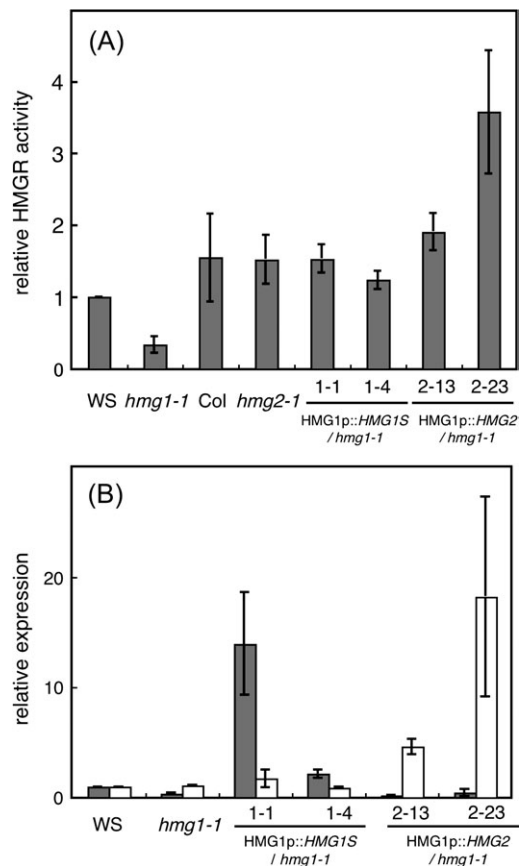


Fig. 2. HMGR enzyme activity and expression of *hmgr* mutants and complementation lines of *hmg1-1*. (A) Relative HMGR activity in microsomes prepared from 2-week-old seedlings of WS, *hmg1-1*, Col, *hmg2-1*, lines 1.1 and 1.4, and lines 2.13 and 2.23. The activity in WS plants, 151 ± 6.9 pmol min⁻¹ mg⁻¹ protein, was given a value of 1. Values are the average of three experiments. (B) Relative expression of *HMGI* (gray box) and *HMG2* (white box) in 2-week-old seedlings of WS, *hmg1-1*, lines 1.1 and 1.4 (both have HMGI promoter::*HMG1S* in an *hmg1-1* background), and lines 2.13 and 2.23 (both have HMGI promoter::*HMG2* in an *hmg1-1* background). The expression in WS plants was given a value of 1.

expression in line 1.4 was comparable to that of WT plants (Fig. 2B). Low levels of *HMGI* expression were detected in *hmg1-1* (Fig. 2B). The primer set of *HMGI* for this quantitative RT-PCR was designed prior to the T-DNA insertion. Northern hybridization showed no transcript for *HMGI* in *hmg1-1* (Suzuki *et al.*, 2004). T-DNA was inserted before the catalytic domain of HMGR1. Although small amounts of transcript derived from *HMGI* were detected in *hmg1-1*, it is considered that no functional HMGR1 exists in *hmg1-1*. The expression of *HMG2* in lines 2.13 and 2.23 was much higher than that of WT plants (Fig. 2B). The HMGR assay demonstrated that the HMGR activity of transgenic lines was comparable to or higher than that of WT plants (Fig. 2A).

The expression of *HMG2* driven by the HMGI promoter complemented the characteristic *hmg1* phenotype in seedlings, such as the inhibition of root growth, small

cotyledons, and delayed development of rosette leaves, similar to the expression of *HMG1S* (data not shown). In mature plants, the height and fertility of *hmg1-1* plants expressing *HMG2* were comparable to those of WT plants and of *hmg1-1* plants expressing *HMG1S* (Fig. 3A). Because sterols are the major products of the MVA pathway, the total amount of sterols in *hmg1* plants is approximately 50% that of WT plants (Suzuki *et al.*, 2004; Ohyama *et al.*, 2007). To test whether *HMG2* expression in *hmg1-1* rescues the sterol level, the sterols were quantified. The total amount of sterols in *hmg1-1* was restored to that of WT plants and of *hmg1-1* plants expressing *HMG1S* by the expression of *HMG2* under the control of the *HMG1* promoter (Fig. 3B). The sterol profile was not altered in these transgenic plants (data not shown).

The expression of *XTR9* and *extensin-like protein*, which are thought to be related to cell elongation, is lower in *hmg1* plants than in WT plants (Suzuki *et al.*, 2004). The expression of these genes in transgenic *hmg1-1* plants was examined. The expression of both genes down-regulated in *hmg1* plants was restored, with expression down-regulated to that in WT plants, in transgenic *hmg1-1* plants expressing *HMG2* or *HMG1S* (Fig. 3C). The complementation of the *hmg1* mutation by *HMG2* indicates that the biochemical and cellular functions of HMGR2 are equal to those of HMGR1.

An *hmg1 hmg2* double mutant was not obtained

As mentioned above, two genes encoding functional HMGR isoforms occur in the *Arabidopsis* genome. To examine the contribution of the MEP pathway to the biosynthesis of cytosolic isoprenoids, an attempt was made

to generate a *hmg1-1 hmg2-1* double mutant that completely lacked MVA pathway activity. However, a double homozygous mutant was not produced, even in the progeny of self-pollinated *HMG1/hmg1-1 hmg2-1/hmg2-1* plants.

To determine why double mutants were not obtained, crossing experiments were performed. An *hmg1-1* homozygous mutant was obtained from the progeny of self-pollinated *hmg1-1* heterozygous parents in the expected 1:2:1 Mendelian segregation ratio (Fig. 4A). This indicates that *hmg1-1* *HMG2* male gametophytes were functional in *hmg1-1* heterozygous parents. If *HMG1* deficiency is a gametophytic mutation that affects the haploid life cycle, segregation distortion should be evident. It has already been shown that *hmg1* homozygous plants exhibit male sterility (Suzuki *et al.*, 2004). Because the segregation was not distorted, *HMG1* deficiency of male sterility in *hmg1* homozygous plants is considered to be a sporophytic mutation that affects diploid cells such as tapetum cells or microsporocytes.

There are two *hmg1* alleles: *hmg1-1* and *hmg1-2* (Suzuki *et al.*, 2004). When *HMG1/hmg1-2 hmg2-1/hmg2-1* plants were pollinated with pollen from *HMG1/hmg1-1 hmg2-1/hmg2-1* plants, 98 plants with *hmg2-1* homozygous single mutants and 78 plants with *HMG1/hmg1-2 hmg2-1/hmg2-1* were obtained in the F₁ generation (Fig. 4B). No *HMG1/hmg1-1 hmg2-1/hmg2-1* plants or *hmg1 hmg2* double homozygotes were obtained. When *HMG1/hmg1-1 hmg2-1/hmg2-1* plants were pollinated with pollen from *HMG1/hmg1-2 hmg2-1/hmg2-1* plants, four plants with *hmg2-1* homozygous single mutants and four plants with *HMG1/hmg1-1 hmg2-1/hmg2-1* were obtained in the F₁ generation. No *HMG1/hmg1-2 hmg2-1/hmg2-1* plants or *hmg1 hmg2* double homozygotes were obtained. These crossing experiments

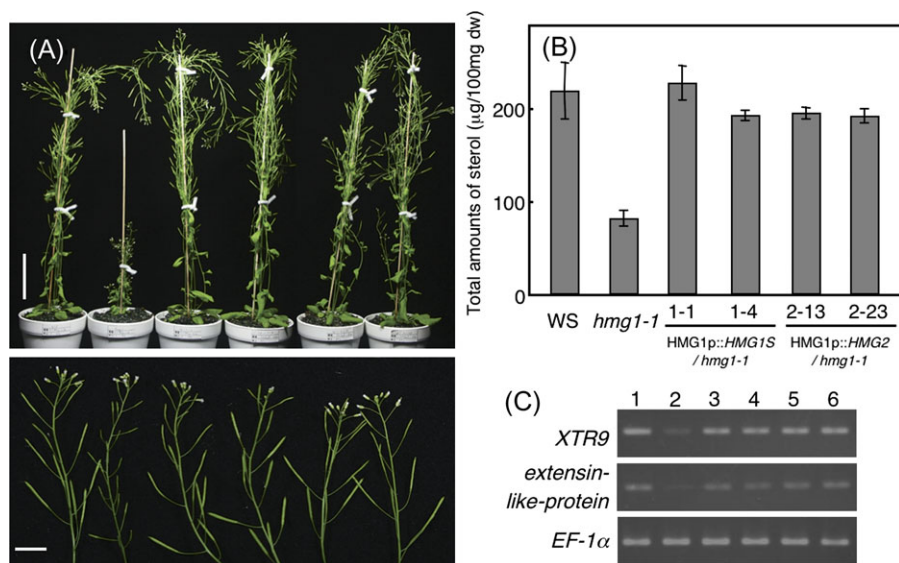


Fig. 3. Phenotypic analyses of *hmg1-1* complementation lines. (A) Left to right, upper figure: mature WS, *hmg1-1*, 1.1, 1.4, 2.13, and 2.23 plants. Scale bar indicates 5 cm. Left to right, lower figure: close-up photos of inflorescences of WS, *hmg1-1*, 1.1, 1.4, 2.13, and 2.23 plants. Scale bar indicates 1 cm. (B) Total sterols ($\mu\text{g } 100\text{mg}^{-1}$ dry weight) in WS, *hmg1-1*, 1.1, 1.4, 2.13, and 2.23 plants. (C) The effect of *HMG1S* and *HMG2* expressed in *hmg1-1* plants on *XTR9* and *extensin-like-protein* expression. Total RNA was extracted from 2-week-old seedlings of WS (1), *hmg1-1* (2), 1.1 (3), 1.4 (4), 2.13 (5), and 2.23 (6) plants. *EF-1* transcripts were amplified as a control.

demonstrated that the *hmg1* mutation in the F₁ generation was inherited only from the female parent. These results suggest that the *hmg1 hmg2* male gametophytes were lethal and that the genotype could not be inherited from the male parent. While *HMG1* deficiency in *hmg1* homozygotes

sporophytically cause male sterility, double deficiency of *HMG1* and *HMG2* may gametophytically cause defects of the male gametophyte.

hmg1 hmg2 male gametophytes are shrunken

To examine the influence of the *hmg1 hmg2* double mutation on the male gametophyte, the anthers of *HMG1/HMG1 hmg2-1/hmg2-1* and *HMG1/hmg1-1 hmg2-1/hmg2-1* plants were compared microscopically just before flowering. Although all pollen grains were globular in *HMG1/HMG1 hmg2-1/hmg2-1* anthers, approximately half of the pollen grains were shrunken in *HMG1/hmg1-1 hmg2-1/hmg2-1* anthers (Fig. 5A, B). The hypothesis that the shrunken pollen grains correspond to those of the *hmg1 hmg2* genotype is consistent with the crossing data. To confirm this, tetrad analysis was attempted using *quartet1* (*qrt1*) (Preuss et al., 1994). In microsporogenesis, a microsporocyte becomes a tetrad of four haploid cells after meiosis. The tetrad is then separated into four microspores, which develop into mature male gametophytes. In the *qrt1* mutant, the tetrad does not separate during male gametophyte development (Preuss et al., 1994). If HMGR deficiency is sporophytically expressed, the numbers of normal and shrunken pollen might vary in a given quartet. By contrast, if the mutation is gametophytic, the ratio of normal to shrunken pollen in each quartet should be 2:2. To determine the genotype of these shrunken pollen grains, an *HMG1/hmg1-1 hmg2-1/hmg2-1 qrt1-1/qrt1-1* triple mutant was generated, and tetrad analysis using this triple mutant was performed. Tetrads of *qrt1-1* single mutants produced four normal pollen grains (Fig. 5C). By contrast,

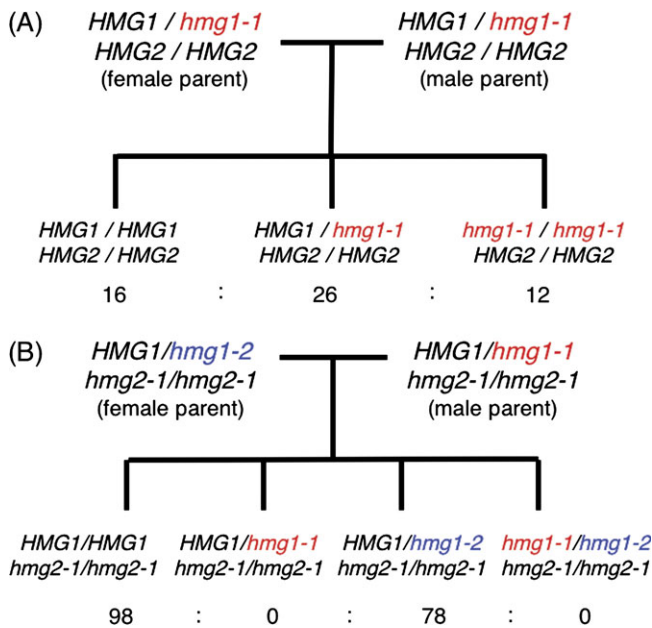


Fig. 4. Outline of the crossing experiment. The results of self-pollination of *HMG1/hmg1-1 HMG2/HMG2* plants (A) and crossing of *HMG1/hmg1-2 hmg2-1/hmg2-1* as the female parent pollinated with pollen from *HMG1/hmg1-1 hmg2-1/hmg2-1* plants (B) are shown. The resultant segregation of the F₁ generation is shown.

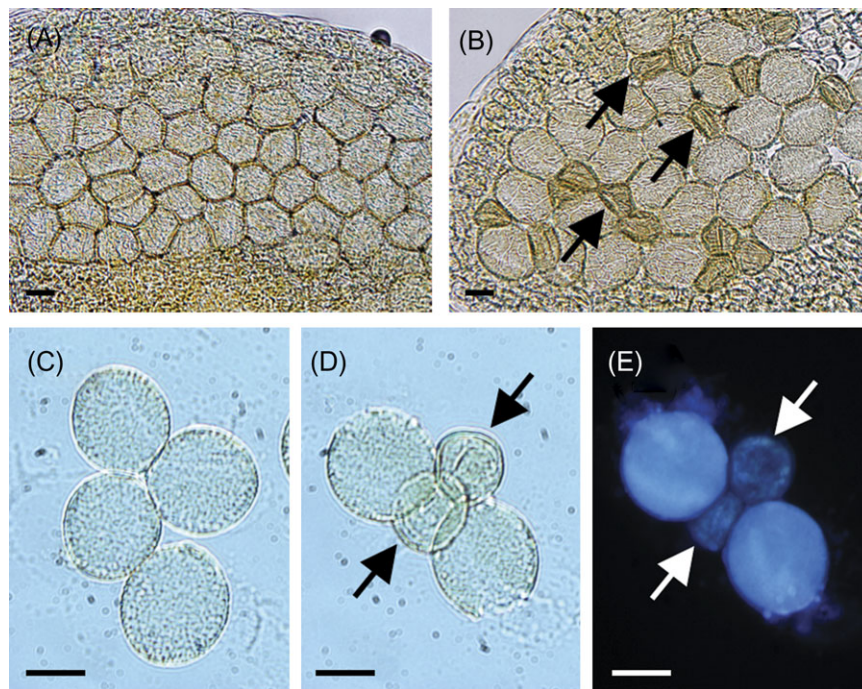


Fig. 5. Photographs of the anthers and pollen grains. (A) Anther of *HMG1/HMG1 hmg2-1/hmg2-1* plant. (B) Anther of a *HMG1/hmg1-1 hmg2-1/hmg2-1* plant. (C) Tetrad in *HMG1/HMG1 HMG2/HMG2 qrt1-1/qrt1-1* plant. (D) Tetrad in *HMG1/hmg1-1 hmg2-1/hmg2-1 qrt1-1/qrt1-1* plant. (E) DAPI-stained tetrad shown in (D). Arrows indicate abnormal, shrunken pollen grains. Scale bars indicate 10 μm.

tetrads of *HMG1/hmg1-1 hmg2-1/hmg2-1 qrt1-1/qrt1-1* triple mutants produced two normal and two shrunken pollen grains (Fig. 5D). No nuclei were observed in DAPI-stained shrunken pollen grains (Fig. 5E). These results strongly suggest that the two shrunken pollen grains were *hmg1-1 hmg2-1 qrt1-1* and that the male gametophyte of the *hmg1 hmg2* genotype exhibits lethality, whereas that of single *hmg2* does not.

To determine why the *hmg1 hmg2* pollen grains were shrunken, the ultrastructure of the male gametophyte was next observed at the tricellular stage in *HMG1/hmg1-1 hmg2-1/hmg2-1* plants using a transmission electron microscope. The inside of the globular male gametophyte was densely packed, and its cytoplasm, organelles, exine, and pollen coat were perfectly normal (Fig. 6A), whereas the inside of the shrunken male gametophyte was hollow and less packed (Fig. 6B, C, D). However, the exine and pollen coat were normal in the shrunken male gametophyte. Magnified views of the shrunken male gametophyte showed that the vacuole was larger than normal, and the membranous structures that seemed to originate from the endo-

plasmic reticulum (ER) were hypertrophic (Fig. 6E, F, G), whereas symbiotic organelles (plastids and mitochondria) appeared nearly normal (Fig. 6G, H, I). The most remarkable aberration in shrunken male gametophytes is the poor condition of the membranous element in the membrane traffic system. Thus, HMGR is essential for the development of male gametophytes, especially the formation of membranes originating from the ER. This indicates that the contribution of metabolic cross-talk from the MEP pathway is not very effective in male gametophyte development.

HMG1 and HMG2 are expressed in microspores

The spatial distribution of *HMG1* and *HMG2* in WT anthers was examined using *in situ* hybridization. *HMG1* expression was observed in both the tapetum and microspores. *HMG2* expression was detected only in the microspores (Fig. 7). Thus, both *HMG1* and *HMG2* genes are expressed in microspores. In contrast to the double mutant (microspores with a *hmg1 hmg2* genotype), the microspores in *hmg1* and *hmg2* plants develop normally. In the single mutant, the

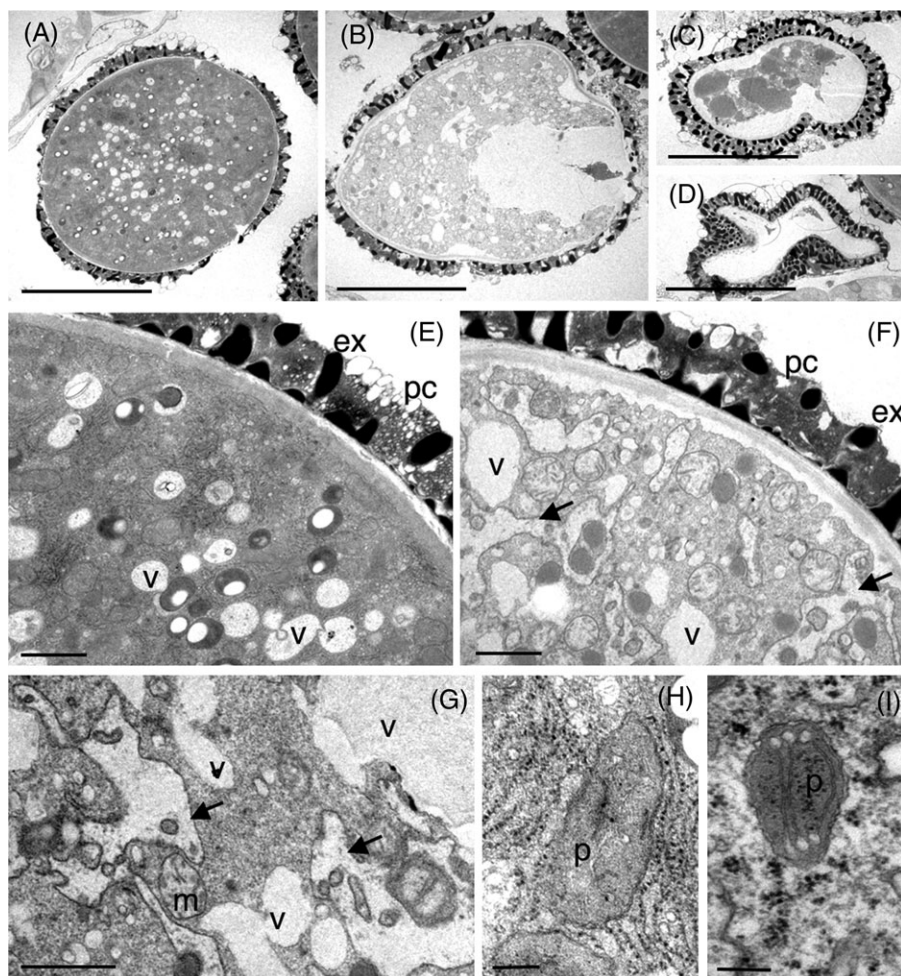


Fig. 6. Ultrastructural analysis of the male gametophyte. Normal male gametophytes (A, E, H) and abnormal male gametophytes (B, C, D, F, G, I) in *HMG1/hmg1-1 hmg2-1/hmg2-1* plants. Abbreviations: ex, exine; pc, pollen coat; v, vacuole; m, mitochondria; p, plastid. Arrows indicate abnormal membrane structures of apparent endoplasmic reticulum origin. Scale bars indicate 10 μm (A, B, C, D), 1 μm (E, F, G), or 200 nm (H, I).

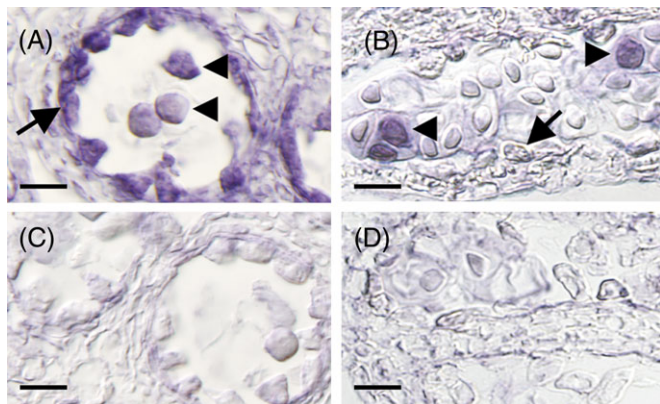


Fig. 7. Comparison of *HMG1* and *HMG2* expression in anthers using *in situ* hybridization. Sections of an anther hybridized with antisense *HMG1* (A) or *HMG2* (B) probes. Sections of an anther hybridized with sense *HMG1* (C) or *HMG2* (D) probes as a control. Arrows and arrowheads indicate the tapetum and microspores, respectively. Scale bars indicates 10 μm .

remaining HMGR may compensate for one HMGR mutation during microspore and male gametophyte development.

Discussion

Since the discovery of the MEP pathway, metabolic cross-talk between the cytosol and plastid has been a central topic in isoprenoid biosynthesis research. Despite numerous endeavours, it has remained unclear whether native cross-talk, rather than exogenously applied metabolites, can compensate for the complete blockage of one of the isoprenoid biosynthetic pathways.

The biochemical and cellular functions of HMGR2 were analysed and the *hmg1-1 hmg2-1* double mutant was characterized to assess the contribution of the native metabolic flow from the MEP pathway. The *hmg1-1* plants have approximately 20% of the activity of WT plants, which is thought to correspond to HMGR2 activity (Fig. 2A). *HMG2* expression under an *HMG1* promoter complemented the *hmg1* phenotype in terms of HMGR activity, sterol accumulation, gene expression, and morphology (Fig. 3). Although the physiological function(s) of *HMG1* and *HMG2* differ from each other (because of the difference in the phenotypes of *hmg1* and *hmg2*), it was demonstrated that *HMG2* encodes a functional HMGR. Although *HMG1* expression is not affected in *hmg2* plants (Suzuki *et al.*, 2004), the HMGR activity of *hmg2-1* plants was comparable to that of WT plants. HMGR activity of transgenic *hmg1-1* lines with *HMG1* or *HMG2* genes was not necessarily parallel to the expression level of *HMG1* or *HMG2* genes in these lines. These results suggest that HMGR activity is post-transcriptionally and post-translationally regulated. Post-transcriptional and post-translational feedback regulation of HMGR was suggested by the experiments using *Arabidopsis* treated with lovastatin (Kobayashi *et al.*,

2007) and with squalastatin and terbinafine, inhibitors of squalene synthase and of squalene epoxidase, respectively (Nieto *et al.*, 2009). The feedback regulation was also suggested by the experiment using tobacco BY-2 cells treated with squalastatin and terbinafine (Wentzinger *et al.*, 2002). Genetic studies have also demonstrated the regulatory mechanisms of *Arabidopsis* HMGR (Rodríguez-Concepción *et al.*, 2004; Kobayashi *et al.*, 2007). Since the occurrence of multiple genes encoding HMGR is a general feature of higher plants, elucidation of the regulatory mechanisms is a complicated but challenging research area.

Because HMGR2 activity was confirmed, an attempt was made to generate an *hmg1-1 hmg2-1* double mutant. However, double homozygotes were not obtained, due to a defect in the male gametophyte of the *hmg1 hmg2* genotype (Fig. 4B). A tetrad analysis of *HMG1/hmg1-1 hmg2-1/hmg2-1* in a *qrt1-1* background demonstrated that the male gametophyte of the *hmg1 hmg2* genotype was shrunken and lethal (Fig. 5). Ultrastructural observations revealed that these disrupted male gametophytes are hollow and that ER-derived membranes are in a poor condition (Fig. 6). The deficiency in male gametophytes might be caused by a defect in the build-up of membrane systems that depend on essential components such as sterols, or on the presence of MVA-derived storage lipids like sterol esters. In contrast to the ER-derived membrane, plastids in shrunken gametophytes appeared normal, which may suggest that blockage of the MVA pathway does not affect plastid morphology. Furthermore, metabolic flow from the MEP pathway in the plastids cannot rescue deficiency of the male gametophyte in the blockage of the MVA pathway, although the plastid appeared normal.

Interestingly, the crossing experiment suggested that complete blockage of the MVA pathway does not affect female gametophyte development (Fig. 4B). It is possible that a sufficient amount of MVA or MVA-derived metabolites is accumulated in the embryo-sac cell after meiosis during female gametophyte development. It is also possible that native cross-talk of isoprenoid compounds from the plastid might be able to compensate for the blockage of the MVA pathway in the embryo-sac cell. Recently, it was shown that complete deficiency of cycloartenol synthase 1 (CAS1) leads to male gametophyte lethality (Babiychuk *et al.*, 2008). Since the embryo-sac cell of the *cas1-2* mutant is viable, the viability of the *hmg1 hmg2* embryo-sac cell may not be derived from the native cross-talk from the plastid. Although important genes (e.g. housekeeping genes) are generally required for both male and female gametophyte development, it is interesting that HMGR, a key enzyme for cytosolic isoprenoid biosynthesis, is required only for male gametophyte development.

It is also interesting the *HMG1* deficiency in *hmg1* homozygous plants is a sporophytic mutation, while complete blockage of *HMGGR* causes a gametophytic mutation. This may be caused by a difference in the expression pattern of *HMG1* and *HMG2* (Fig. 7). In male gametophyte development, both *HMG1* and *HMG2* are expressed, such

that at least one HMGR isoenzyme is required. By contrast, diploid tapetum cells, which are essential for normal pollen formation, express only *HMGI*, so that *hmg1* homozygous plants may exhibit male sterility.

Since prenyl diphosphates are thought to flow between the cytosol and plastids (Bick and Lange, 2003), HMGR is located upstream of the cross-talk in the MVA pathway (Fig. 1). Our data demonstrated that plants completely lacking the MVA pathway did not survive, even if they could biosynthesize small amounts of cytosolic isoprenoids using IPP and DMAPP from plastids. This indicates that the metabolic flow from the MEP pathway is insufficient, at least during male gametophyte development, to compensate for a non-functional MVA pathway. This may reflect the fact that the cytosol and plastid originated from different organisms, a primitive eukaryote and a cyanobacterium, respectively. Even if the contribution of native cross-talk is low, the regulatory mechanisms for isoprenoid biosynthesis in different organelles are interesting and challenging research areas.

Acknowledgements

We thank Dr. Hikaru Seki (RIKEN, PSC) for helpful discussions, and Mizuki Magoshiro, Misaki Yano, Chiho Yamazaki, and Naomi Suzuki for technical assistance with *in situ* hybridization and TEM. This work was supported in part by a Grant-in-Aid for Young Scientists (B) (grant 18770048), a Scientific Research on Priority Areas grant (grant 17051028) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Research and Development Program for New Bio-industry Initiatives, Japan.

References

- Alberts AW, Chen J, Kuron G, et al.** 1980. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proceedings of the National Academy Sciences, USA* **77**, 3957–3961.
- Araki N, Kusumi K, Masamoto K, Niwa Y, Iba K.** 2000. Temperature-sensitive *Arabidopsis* mutant defective in 1-deoxy-D-xylulose 5-phosphate synthase within the plastid non-mevalonate pathway of isoprenoid biosynthesis. *Physiologia Plantarum* **108**, 19–24.
- Babiychuk E, Bouvier-Navé P, Compagnon V, Suzuki M, Muranaka T, Montagu MV, Kushnir S, Schaller H.** 2008. Allelic mutant series reveal distinct functions for *Arabidopsis* cycloartenol synthase 1 in cell viability and plastid biogenesis. *Proceedings of the National Academy Sciences, USA* **105**, 3163–3168.
- Bach TJ, Lichtenthaler HK.** 1987. Plant growth regulation by mevinolin and other sterol biosynthesis inhibitors. In: Fuller G, Nes WD, eds. *Ecology and metabolism of plant lipids*. American Chemical Society Symposium Ser. 325. Washington: American Chemical Society, 109–139.
- Bach TJ, Weber T, Motel A.** 1990. Some properties of enzymes involved in the biosynthesis and metabolism of 3-hydroxy-3-methylglutaryl-CoA in plants. *Recent Advances in Phytochemistry* **24**, 1–82.
- Bick JA, Lange BM.** 2003. Metabolic cross-talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. *Archives of Biochemistry and Biophysics* **415**, 146–154.
- Ceccarelli N, Lorenzi R.** 1984. Growth inhibition by competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase in *Helianthus tuberosus* tissue explants. *Plant Science Letters* **34**, 269–276.
- Chappell J, Wolf F, Proulx J, Cuellar R, Saunders C.** 1995. Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants? *Plant Physiology* **109**, 1337–1343.
- Crowell DN, Salaz MS.** 1992. Inhibition of growth of cultured tobacco cells at low concentrations of lovastatin is reversed by cytokinin. *Plant Physiology* **100**, 2090–2095.
- Dale S, Arró M, Becerra B, Morrice NG, Boronat A, Hardie DG, Ferrer A.** 1995. Bacterial expression of the catalytic domain of 3-hydroxy-3-methylglutaryl-CoA reductase (isoform HMGR1) from *Arabidopsis thaliana*, and its inactivation by phosphorylation at Ser577 by *Brassica oleracea* 3-hydroxy-3-methylglutaryl-CoA reductase kinase. *European Journal of Biochemistry* **233**, 506–513.
- Estévez JM, Cantero A, Romero C, Kawaide H, Jiménez LF, Kuzuyama T, Seto H, Kamiya Y, León P.** 2000. Analysis of the expression of *CLA1*, a gene that encodes the 1-deoxyxylulose 5-phosphate synthase of the 2-C-methyl-D-erythritol-4-phosphate pathway in *Arabidopsis*. *Plant Physiology* **124**, 95–103.
- Guevara-García A, Roman CS, Arroyo A, Cortés ME, Gutiérrez-Nava MDL, León P.** 2005. Characterization of *Arabidopsis clb6* mutant illustrates the importance of post-transcriptional regulation of the methyl-D-erythritol 4-phosphate pathway. *The Plant Cell* **17**, 628–643.
- Gutiérrez-Nava MDL, Gillmor CS, Jiménez LF, Guevara-García A, León P.** 2004. *CHLOROPLAST BIOGENESIS* genes act cell and noncell autonomously in early chloroplast development. *Plant Physiology* **135**, 471–482.
- Hata S, Shirata K, Takagishi H, Kouchi H.** 1987. Accumulation of rare phytosterols in plant cells on treatment with metabolic inhibitors and mevalonic acid. *Plant and Cell Physiology* **28**, 715–722.
- Hemmerlin A, Bach TJ.** 1998. Effects of mevinolin on cell cycle progression and viability of tobacco BY-2 cells. *The Plant Journal* **14**, 65–74.
- Hemmerlin A, Hoeffler J-F, Meyer O, Tritsch D, Kagan IA, Grosdemange-Billiard C, Rohmer M, Bach TJ.** 2003. Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. *Journal of Biological Chemistry* **278**, 26666–26676.
- Hsieh M-H, Goodman HM.** 2005. The *Arabidopsis* IspH homolog is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis. *Plant Physiology* **138**, 641–653.
- Hsieh M-H, Goodman HM.** 2006. Functional evidence for the involvement of *Arabidopsis* IspF homolog in the nonmevalonate pathway of plastid isoprenoid biosynthesis. *Planta* **223**, 779–784.

- Kasahara H, Hanada A, Kuzuyama T, Takagi M, Kamiya Y, Yamaguchi S.** 2002. Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in *Arabidopsis*. *Journal of Biological Chemistry* **277**, 45188–45194.
- Kobayashi K, Suzuki M, Tang J, et al.** 2007. LOVASTATIN INSENSITIVE 1, a novel pentatricopeptide repeat protein, is a potential regulatory factor of isoprenoid biosynthesis in *Arabidopsis*. *Plant and Cell Physiology* **48**, 322–331.
- Lange BM, Ghassemian M.** 2003. Genome organization in *Arabidopsis thaliana*: a survey for genes involved in isoprenoid and chlorophyll metabolism. *Plant Molecular Biology* **51**, 925–948.
- Learned RM.** 1996. Light suppresses 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene expression in *Arabidopsis thaliana*. *Plant Physiology* **110**, 645–655.
- Learned RM, Fink GR.** 1989. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase from *Arabidopsis thaliana* is structurally distinct from the yeast and animal enzymes. *Proceedings of the National Academy of Sciences, USA* **86**, 2779–2783.
- Lumbreras V, Campos N, Boronat A.** 1995. The use of an alternative promoter in the *Arabidopsis thaliana* *HMG1* gene generates an mRNA that encodes a novel 3-hydroxy-3-methylglutaryl coenzyme A reductase isoform with an extended N-terminal region. *The Plant Journal* **8**, 541–549.
- Mandel MA, Feldmann KA, Herrera-Estrella L, Rocha-Sosa M, León P.** 1996. *CLA1*, a novel gene required for chloroplast development, is highly conserved in evolution. *The Plant Journal* **9**, 649–658.
- Manzano D, Fernández-Busquets X, Schaller H, González V, Boronat A, Arró M, Ferrer A.** 2004. The metabolic imbalance underlying lesion formation in *Arabidopsis thaliana* overexpressing farnesyl diphosphate synthase (isoform 1S) leads to oxidative stress and is triggered by the developmental decline of endogenous HMGR activity. *Planta* **219**, 982–992.
- Morehead TA, Biermann BJ, Crowell DN, Randall SK.** 1995. Changes in protein isoprenylation during growth of suspension-cultured tobacco cells. *Plant Physiology* **109**, 277–284.
- Nagata N, Suzuki M, Yoshida S, Muranaka T.** 2002. Mevalonic acid partially restores chloroplast and etioplast development in *Arabidopsis* lacking the non-mevalonate pathway. *Planta* **216**, 345–350.
- Newman JD, Chappell J.** 1997. Isoprenoid biosynthesis in plants: carbon partitioning within the cytoplasmic pathway. In: Parish EJ, Nes WD, eds. *Biochemistry and function of sterols*. Boca Raton, FL: CRC Press, 123–134.
- Nieto B, Forés O, Arró M, Ferrer A.** 2009. *Arabidopsis* 3-hydroxy-3-methylglutaryl-CoA reductase is regulated at the post-translational level in response to alterations of the sphingolipid and the sterol biosynthetic pathways. *Phytochemistry* **70**, 58–64.
- Ohyama K, Suzuki M, Masuda K, Yoshida S, Muranaka T.** 2007. Chemical phenotypes of the *hmg1* and *hmg2* mutants of *Arabidopsis* demonstrate the *in planta* role of HMG-CoA reductase in triterpene biosynthesis. *Chemical and Pharmaceutical Bulletin* **55**, 1518–1521.
- Okada K, Kasahara H, Yamaguchi S, Kawaide H, Kamiya Y, Nojiri H, Yamane H.** 2008. Genetic evidence for the role of isopentenyl diphosphate isomerases in the mevalonate pathway and plant development in *Arabidopsis*. *Plant and Cell Physiology* **49**, 604–616.
- Phillips MA, D'Auria JC, Gershenzon J, Pichersky E.** 2008. The *Arabidopsis thaliana* type 1 isopentenyl diphosphate isomerase are targeted to multiple subcellular compartments and gave overlapping functions in isoprenoid biosynthesis. *The Plant Cell* **20**, 677–696.
- Preuss D, Rhee SY, Davis RW.** 1994. Tetrad analysis possible in *Arabidopsis* with mutation of the *QUARTET (QRT)* genes. *Science* **264**, 1458–1460.
- Randall SK, Marshall MS, Crowell DN.** 1993. Protein isoprenylation in suspension-cultured tobacco cells. *The Plant Cell* **5**, 433–442.
- Rohdich F, Kis K, Bacher A, Eisenreich W.** 2001. The non-mevalonate pathway of isoprenoids: genes, enzymes and intermediates. *Current Opinion in Chemical Biology* **5**, 535–540.
- Rodríguez-Concepción M, Forés O, Martínez-García JF, González V, Phillips MA, Ferrer A, Boronat A.** 2004. Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during *Arabidopsis* seedling development. *The Plant Cell* **16**, 144–156.
- Ryder NS, Goad LJ.** 1980. The effect of the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor ML-236 B on phytosterol synthesis in *Acer pseudoplatanus* tissue culture. *Biochimica et Biophysica Acta* **619**, 424–427.
- Sapir-Mir M, Mett A, Belausov E, Tal-Meshulam T, Frydman A, Gidoni D, Eyal Y.** 2008. Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. *Plant Physiology* **148**, 1219–1228.
- Schaller H, Grausem B, Benveniste P, Chye ML, Tan CT, Song YH, Chua NH.** 1995. Expression of the *Hevea brasiliensis* (H.B.K.) Müll. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. *Plant Physiology* **109**, 761–770.
- Schwender J, Gemünden C, Lichtenthaler HK.** 2001. Chlorophyta exclusively use the 1-deoxyxylulose 5-phosphate/2-C-methylerythritol 4-phosphate pathway for the biosynthesis of isoprenoids. *Planta* **212**, 416–423.
- Suzuki M, Kamide Y, Nagata N, et al.** 2004. Loss of function of 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (*HMG1*) in *Arabidopsis* leads to dwarfing, early senescence and male sterility, and reduced sterol levels. *The Plant Journal* **37**, 750–761.
- Suzuki M, Muranaka T.** 2007. Molecular genetics of plant sterol backbone synthesis. *Lipids* **42**, 47–54.
- Wentzinger LF, Bach TJ, Hartmann MA.** 2002. Inhibition of squalene synthase and squalene epoxidase in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Plant Physiology* **130**, 334–346.