Chemical Phenotypes of the *hmg1* and *hmg2* Mutants of *Arabidopsis* Demonstrate the *In-planta* Role of HMG-CoA Reductase in Triterpene Biosynthesis

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Plants produce a wide variety of cyclic triterpenes, such as sterols and triterpenoids, which are the major products of the mevalonate (MVA) pathway. It is important to understand the physiological functions of HMG-CoA reductase (HMGR) because HMGR is the rate-limiting enzyme in the MVA pathway. We have previously isolated *Arabidopsis* mutants in *HMG1* and *HMG2*. Although the biochemical function of HMGR2 has been thought to be almost equal to that of HMGR1, based on similarities in their sequences, the phenotypes of mutants in these genes are quite different. Whereas *hmg2* shows no abnormal phenotype under normal growth conditions, *hmg1* shows pleiotropic phenotypes, including dwarfing, early senescence, and male sterility. We previously postulated that the 50% decrease in the sterol content of *hmg1*, as compared to that in the wild type, was a cause of these phenotypes,[1)] but comprehensive triterpene profiles of these mutants had not yet been determined. Here, we present the triterpene profiles of *hmg1* and *hmg2*. In contrast to *hmg1*, *hmg2* showed a sterol content 15% lower than that of the wild type. A precise triterpenoid quantification using synthesized deuterated compounds of β-amyrin (1), α-amyrin (2), and lupeol (3) showed that the levels of triterpenoids in *hmg1* and *hmg2* were 65% and 25% lower than in the wild type (WT), respectively. These results demonstrate that HMGR2 as well as HMGR1 is responsible for the biosynthesis of triterpenes in spite of the lack of visible phenotypes in *hmg2*.

Key words HMG-CoA reductase; sterol; triterpenoid; synthesis; *Arabidopsis*; amyrin

The plant natural products that are derived from squalene can be divided into two groups: steroids derived from cycloartenol (**4**), such as phytosterols and brassinosteroids, and triterpenoids, such as β-amyrin (**1**) and lupeol (**3**) (Fig. 1). Phytosterols are highly diverse and are important for plant growth and development as structural components of the plasma membrane and biosynthetic intermediates of brassinosteroids. Recently, lanosterol synthases were identified in several plants.[2—4)] Since this new enzyme of sterol backbone synthesis has been identified, the known diversity of phytosterols has increased even more. Triterpenoids are plant-characteristic isoprenoids; they also form a large, structurally diverse group of natural products[5—7)] with more than 100 different carbon skeletons.[8)] Some have a variety of pharmacological activities. In particular, their glycosides are often active constituents of important traditional medicines.[9)] Many studies on the biosynthesis of these compounds have focused on the biosynthesis of brassinosteroids from campesterol.[10,11)] In addition, genes encoding glycosyltransferases, which are involved in steroidal saponin biosynthesis, have been identified.[12)] However, it is not yet known how the majority of these compounds are regulated in response to development or environmental conditions.

Plants biosynthesize isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), common precursors of isoprenoids, *via* two pathways: the cytosolic mevalonate (MVA) pathway and the plastidic 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. The metabolic flow

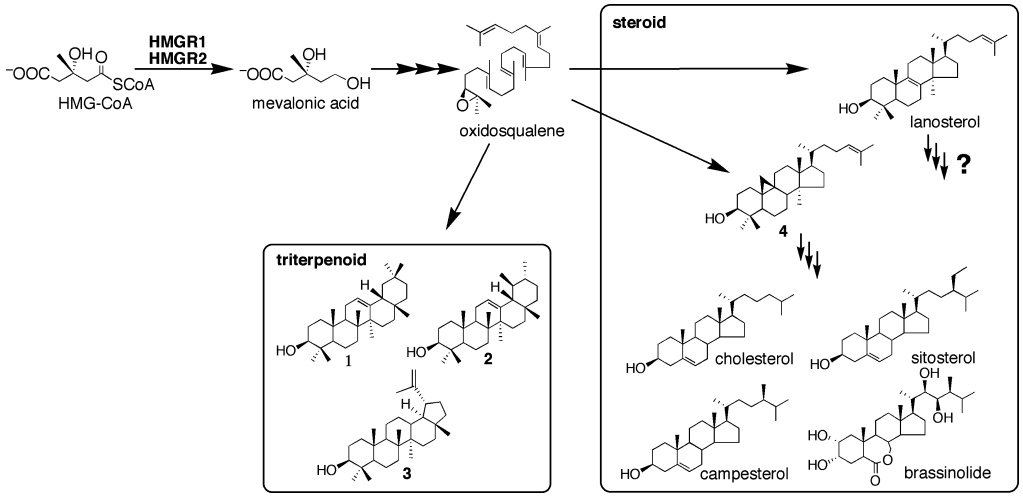

Fig. 1. Biosynthetic Pathways of Steroids and Triterpenoids in Plants

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between these pathways has been recently reported.[13—15] Steroids and triterpenoids, which are the main MVA-derived isoprenoidal endproducts, are biosynthesized from acetyl-CoA at several steps. A few functional analyses of the enzymes responsible for these steps *in planta* have been reported.[16] As HMG-CoA reductase (HMGR) is the rate-limiting enzyme[1,17,18] of the MVA pathway, many plant HMGRs have been characterized. Two genes encoding HMGRs, *HMG1*[19,20] and *HMG2*,[20] exist in the *Arabidopsis thaliana* genome. The structure and expression of the *Arabidopsis HMG1* and *HMG2* genes have been analyzed. To manipulate the synthesis of phytosterols and triterpenoids, and to understand the physiological roles of these compounds, we have isolated and characterized *Arabidopsis* T-DNA insertion mutants in *HMG1* and *HMG2*. The *hmg1* mutant shows dwarfing, male sterility, and early senescence. The sterol levels in *hmg1* are approximately 50% lower than in the WT.[1] In contrast, *hmg2* shows no unusual visible phenotypes under normal growth conditions. To understand why *hmg2* appears normal, it is important that the triterpene contents in these mutants be investigated. In this study, we determined the metabolic profiles of sterols and triterpenoids in WT lines and the mutants. To investigate these chemical profiles using the same plant samples, the sterol profile of *hmg1* was reanalyzed. The triterpenoid levels in the WT (Wassilewskija (WS), Columbia (Col)) and the mutants (*hmg1*, *hmg2*) were determined by GC-MS analysis using synthetic labeled triterpenoids as internal standards.

Experimental

General ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL Alpha-500 spectrometer in $CDCl_3$. Analytical TLC was carried out on Merck silica-gel 60F-254 plates (0.25 mm precoated). Oleanolic acid (**5**), ursolic acid (**6**), betulinic acid (**7**), and lupeol (**3**) were purchased from Sigma. β-Amyrin (**1**) and α-amyrin (**2**) were purchased from Apin Chemicals Limited. $LiAlD_4$ (98% D) and $NaBD_4$ (98% D) were purchased from Acros Organics and Aldrich, respectively. The structures of all of the endogenous triterpenoids in *Arabidopsis* were confirmed by comparing their retention times in GC and MS spectra with those of authentic samples.

Plant Materials Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotypes WS and Col, and the mutants *hmg1* (WS background) and *hmg2* (Col background), were germinated on agar plates containing 1X MS medium (Invitrogen) and 3% sucrose.

[3,28,28,28-$^2H_4$]β-Amyrin (**8**): ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.791 (3H, s, H_3-24), 0.871 (6H, s, H_3-29, -30), 0.938 (3H, s, H_3-25), 0.967 (3H, s, H_3-26), 0.996 (3H, s, H_3-23), 1.134 (3H, s, H_3-27), 5.183 (1H, dd, J=3.5, 3.5 Hz, H-12). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 15.48 (C-25), 15.56 (C-24), 16.80 (C-26), 18.38 (C-6), 23.53 (C-11), 23.70 (C-30), 25.99 (C-27), 26.16 (C-15), 26.89 (C-16), 27.14 (C-2), 28.06 (C-23), 31.09 (C-20), 32.26 (C-17), 32.66 (C-7), 33.35 (C-29), 34.72 (C-21), 36.95 (C-10), 37.05 (C-22), 38.60 (C-1), 38.69 (C-4), 39.80 (C-8), 41.73 (C-14), 46.81 (C-19), 47.17 (C-18), 47.64 (C-9), 55.19 (C-5), 78.50 (t, J=21.63 Hz, C-3), 121.71 (C-12), 145.21(C-13); EI-MS 70 eV (TMS ether), *m/z* (rel. int.): 502 (0.7), 487 (0.3), 397 (0.3), 280 (1.1), 260 (0.4), 246 (0.8), 221 (100), 206 (34), 190 (27).

[28,28,28-$^2H_3$]α-Amyrin (**9**): ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.793 (3H, d, J=5.5 Hz, H_3-29), 0.795 (3H, s, H_3-24), 0.914 (3H, bs, H_3-30), 0.956 (3H, s, H_3-25), 0.999 (3H, s, H_3-23), 1.009 (3H, s, H_3-26), 1.071 (3H, s, H_3-27), 3.224 (1H, dd, J=11.0, 5.0 Hz), 5.17 (1H, dd, J=3.5, 3.5 Hz); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 15.62 (C-24), 15.68 (C-25), 16.85 (C-26), 17.46 (C-29), 18.36 (C-6), 21.40 (C-30), 23.27 (C-27), 23.37 (C-11), 26.63 (C-15), 27.28 (C-2), 28.06 (C-16), 28.13 (C-23), 31.25 (C-21), 32.94 (C-7), 33.53 (C-17), 36.91 (C-10), 38.78 (C-1), 38.80 (C-4), 39.62 (C-20), 39.65 (C-21), 40.02 (C-8), 41.44 (C-22), 42.09 (C-14), 47.73 (C-9), 55.19 (C-5), 59.03 (C-18), 79.04 (C-3), 124.40 (C-12), 139.61 (C-13). EI-MS 70 eV, *m/z* (rel. int.): 501 (1.1), 486 (0.1), 396 (0.4), 279 (1.5), 260 (0.9), 246 (0.3), 234 (0.9), 221 (100), 206 (21), 190 (20).

[28,28,28-$^2H_3$]Lupeol (**10**): ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 0.760 (3H, s, H_3-24), 0.829 (3H, s, H_3-25), 0.944 (3H, s, H_3-27), 0.967 (3H, s, H_3-23), 1.030 (3H, s, H_3-26), 1.680 (3H, s, H_3-30), 1.913 (1H, m, H-21), 2.372 (1H, ddd, J=11.0, 11.0, 6.0 Hz, H-19), 3.186 (1H, dd, J=11.5, 5.0 Hz, H-3), 4.57 (1H, bs, H-29), 4.69 (1H, bs, H-29); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 14.54 (C-27), 15.36 (C-24), 15.97 (C-26), 16.11 (C-25), 18.32 (C-26), 19.30 (C-30), 20.93 (C-11), 25.15 (C-12), 27.41 (C-2), 27.45 (C-15), 27.98 (C-23), 29.85 (C-21), 34.28 (C-7), 35.50 (C-16), 37.17 (C-10), 38.06 (C-13), 38.71 (C-1), 38.85 (C-4), 39.91 (C-22), 40.83 (C-8), 42.76 (C-14), 42.82 (C-17), 47.99 (C-19), 48.28 (C-18), 50.44 (C-9), 55.30 (C-5), 78.98 (C-3), 109.30 (C-20), 150.96 (C-29). EI-MS 70 eV, *m/z* (rel. int.): 501 (7.0), 486 (1.9), 411 (1.4), 396 (2.3), 372 (4.9), 328 (1.0), 279 (4.7), 260 (3.0), 248 (2.1), 234 (9.1), 221 (27), 206, (36), 189 (100).

Tissue Extraction and GC-MS Quantification of Triterpenoids Freeze-dried plant tissues were extracted three times with $CHCl_3$–MeOH (1 : 1). [3,28,28,28-$^2H_4$]β-Amyrin (**8**), [28,28,28-$^2H_3$]α-amyrin (**9**), and [28,28,28-$^2H_3$]lupeol (**10**) (4 μg g^{-1} dry wt. in each triterpenoid) were used as internal standards and added directly to the sample homogenate. The extracts were dried and chromatographed on a silica-gel cartridge column (Sep-Pak® Vac 500 mg, Waters) with hexane–EtOAc (2 : 1) and $CHCl_3$–MeOH (1 : 1). The hexane–EtOAc eluent was dried and saponified with 1 ml each of MeOH and 20% KOH aq. for 1 h at 80 °C. The $CHCl_3$–MeOH eluent was dried in a rotary evaporator. The residue and the extraction debris were combined and hydrolyzed with 1 ml each of MeOH and 4 N HCl for 1 h at 80 °C. These reaction mixtures were then extracted three times with 2 ml of hexane, and the combined hexane layer was evaporated to dryness. The residue was separated on silica-gel preparative TLC plates developed twice with hexane–EtOAc (5 : 1) to give the triterpenoid fraction. The triterpenoid fraction was trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide at 80 °C for 30 min and analyzed using GC-MS. GC-MS analysis was carried out on a mass spectrometer (JMS-AM SUN200, JEOL) connected to a gas chromatograph (6890A, Agilent Technologies) with an Rtx-5MS capillary column (15 m×0.25 mm, 0.25-μm film thickness, Restek). The analytical conditions are as follows: EI (70 eV), source temperature 250 °C, injection temperature 250 °C, column temperature program: 80 °C for 1 min, then raised to 280 °C at a rate of 10 °C/min, and held at this temperature for 15 min; interface temperature 280 °C, carrier gas He, flow rate 1 ml/min, splitless injection. The levels of endogenous **1** and **2** were calculated from the peak area ratios of *m/z* 221 for the internal standards and *m/z* 218 for the endogenous compounds. The level of endogenous **3** was calculated from the peak area ratios of molecular ions for **10** and endogenous **3**.

Results and Discussion

Quantification of Sterols in *hmg1* and *hmg2* The sterols in two-week-old seedlings of WT lines and the mutants were quantified as described.[1] GC chromatogram of sterol fraction in WT (Col) was shown in Fig. 2 representatively. The sterol content of the *hmg2* mutant was approximately 15% lower than that of the WT (Table 1). This result indicates that a 15% decrease in sterols does not lead to visible phenotypes. The sterol levels in *hmg2* were much greater than those in *hmg1*. This result corresponds to the finding that the expression of *HMG1* is much higher than that of *HMG2*.[21] As shown in Table 1, neither the *hmg2* nor the *hmg1* mutation affects the sterol composition. The HMGR activity has no influence on the composition of sterols but affects, instead, the total sterol content.

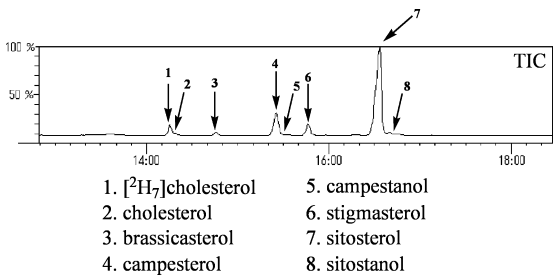

Fig. 2. GC Chromatogram of Sterol Fraction in the WT (Col) of *Arabidopsis*

[$^2H_7$]cholesterol was used as an internal standard.

Synthesis of Deuterated Triterpenoid Internal Standards The triterpenoid contents in *hmg1* and *hmg2* were determined. For standards, we synthesized deuterated triterpenoids, as stable isotope-labeled triterpenoids suitable for use as internal standards in mass spectrometry analysis were not available commercially. As β-amyrin (**1**) and α-amyrin (**2**) had been identified in *Arabidopsis* leaf and stem epicuticular waxes,[22] and **1**, **2**, and lupeol (**3**) had been identified in *Arabidopsis* callus and rosette leaves,[23] we synthesized deuterated triterpenoids of these compounds. The syntheses of [2,2,3-$^2H_3$]β-amyrin[24] and [28,28-$^2H_2$]β-amyrin[25] have been reported. When using isotope-labeled triterpenoids for quantitative GC-MS analysis, the position of the isotope label is important. Triterpenoids with a Δ^{12} double bond, such as **1** and **2**, undergo a retro-Diels-Alder cleavage to form two fragment ions containing the ABC* and C*DE rings (C* indicates the presence of a portion of ring C only) using electron impact ionization.[26] As the most abundant fragment ion in the mass spectra of **1** and **2** appears at *m*/*z* 218, due to the C*DE rings, the isotope label must be on rings D or E. Therefore, we referred to and modified the [28,28-$^2H_2$]β-amyrin synthesis method, and began our synthesis from oleanolic acid (**5**). The synthesis of [3,28,28,28-$^2H_4$]β-amyrin (**8**) is outlined in Fig. 3. We also synthesized deuterated **2** and **3** from ursolic acid (**6**) and betulinic acid (**7**), respectively, using a similar synthesis strategy. Unambiguous assignments of the ^1H and ^{13}C chemical shifts of **1**, **2**, and **3** were confirmed by 2D NMR analysis. A comparison with the NMR and MS spectra of the parent triterpenoids (**1**—**3**) confirmed the chemical structures of each synthetic compound (**8**—**10**).

Table 1. Quantification of Endogenous Sterols and Triterpenoids in two WT Accessions and the *hmg1* and *hmg2* Mutants

Sterols (μg/100 mg dry weight)	WT (WS)	*hmg1*	WT (Col)	*hmg2*
Cholesterol	4.65 (0.44)	3.46 (0.53)	2.21 (0.02)	1.99 (0.16)
Campesterol	44.2 (2.99)	27.6 (1.39)	48.2 (2.75)	41.5 (0.76)
Stigmasterol	19.5 (2.48)	5.23 (0.25)	20.8 (1.08)	21.2 (0.97)
Sitosterol	155 (5.46)	72.1 (6.13)	239 (16.0)	194 (8.13)
Brassicasterol	4.78 (0.20)	1.33 (0.16)	4.15 (0.33)	2.94 (0.11)
Campestanol	0.68 (0.18)	0.39 (0.08)	0.73 (0.04)	0.31 (0.04)
Sitostanol	2.15 (0.67)	1.15 (0.13)	3.55 (0.20)	2.66 (0.03)
Total	231 (11.1)	111 (7.84)	319 (19.7)	264 (10.4)

Triterpenoids (ng/100 mg dry weight)	WT (WS)	*hmg1*	WT (Col)	*hmg2*
1	215 (2.74)	86.1 (9.02)	178 (6.00)	128 (2.65)
2	77.5 (0.39)	36.4 (2.80)	62.2 (0.86)	46.2 (2.74)
3	172 (13.8)	34.1 (2.01)	138 (9.12)	100 (6.64)
Total	465 (16.9)	157 (12.2)	378 (9.57)	275 (6.05)

Values are averages from three samples. Values in parentheses are standard deviations.

Quantification of Triterpenoids in *hmg1* and *hmg2* The triterpenoid levels in two-week-old seedlings of WT lines (ecotype WS, Col) and the mutants were determined using the synthetic compounds as internal standards. The seedlings were extracted with a $CHCl_3$–MeOH solution, and the extracts were subjected to chromatography. Esterified and glycosylated fractions were hydrolyzed with KOH and HCl, respectively. All free triterpenoids were analyzed using GC-MS after conversion to a trimethylsilyl derivative. GC chromatogram of triterpenoid fraction in WT (Col) was shown in Fig. 4 representatively. The levels of endogenous **1** and **2** were calculated from the peak area ratios of *m*/*z* 221 for the internal standards and *m*/*z* 218 for the endogenous compounds. The level of endogenous **3** was calculated from the peak area ratios of molecular ions for trideuterated **3** and endogenous **3**. As shown in Table 1, β-amyrin (**1**), α-amyrin (**2**), and lupeol (**3**) were found in WT seedlings. This is consistent with the report of Husselstein-Muller *et al.*[23] Although these three triterpenoids were also found in the mutants, the levels of these triterpenoids in the *hmg1* and *hmg2* mutants were approximately 65% and 25% lower than in the WT, respectively. These results suggest that both HMGR1 and HMGR2 participate in triterpenoid biosynthesis. These results were first observed experimentally. The decrease in the level of **3** in *hmg1* was greater than that of **1** and **2**. Eleven oxidosqualene cyclases are involved in triterpenoid biosynthesis in *Arabidopsis*. To elucidate the cause of the varying decreases in the levels of individual triterpenoids, the enzymes should be analyzed comprehensively. It is interesting to note that the decrease in the sterol levels was lower than that of the triterpenoid levels. Although triterpenoids are secondary metabolites, sterols are indispensable compounds because they are constituents of the cell membrane and biosynthetic intermediates of phytohormones, and are involved in cell maintenance. It is possible that the restricted amounts of squalene are cyclized into sterols in preference to triterpenoids in the *hmg1* and *hmg2* mutants.

HMGR2 Functions Similarly to HMGR Since the phenotype of the *hmg2* mutant is like that of the WT, it was possible that the sterol and triterpenoid contents of *hmg2* were also similar to those of the WT. However, the levels of sterols and triterpenoids in *hmg2* were approximately 15% and 25% lower than in the WT, respectively. The significant

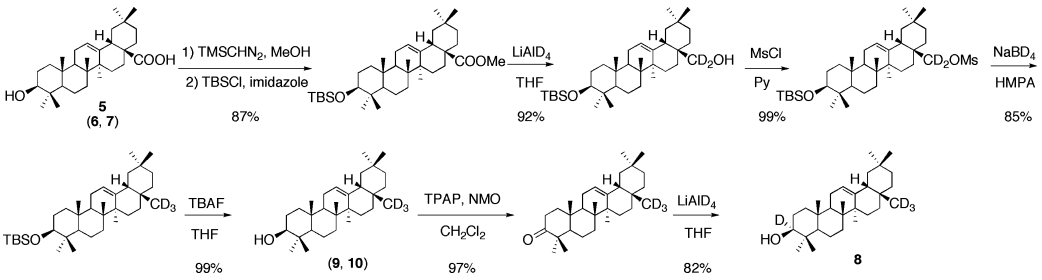

Fig. 3. Total Synthesis of [3,28,28,28-$^2H_4$]β-Amyrin (**8**)

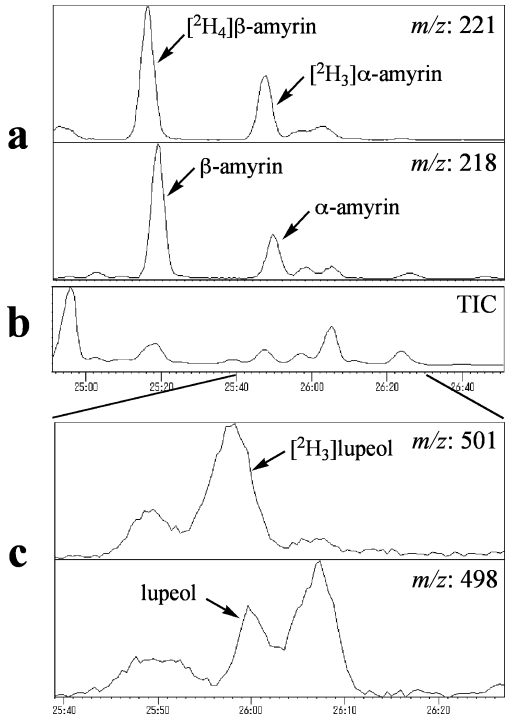

Fig. 4. GC Chromatogram of Triterpenoid Fraction in the WT (Col) of *Arabidopsis*

(a) Mass chromatograms of *m/z* 218 and 221 for amyrins quantifications. (b) Total ion chromatogram of triterpenoid fraction. (c) Mass chromatogram of *m/z* 498 and 501 for lupeol quantification. Deuterated triterpenoids synthesized in this study were used as internal standards.

decreases in these compounds did not affect the morphology of the plants, meaning that plants produce amounts of triterpenes in excess of those required for normal growth. It has been proposed that HMGR2 functions similarly to HMGR in *Arabidopsis*, based on its sequence similarity with HMGR1 and the higher lovastatin sensitivity of *hmg2*.[1)] Our data strongly suggest that HMGR2 functions similarly to HMGR and contributes to triterpene biosynthesis *in planta*.

We are using two approaches leading to a comprehensive understanding of triterpene biosynthesis. One is the analysis of T-DNA insertion mutants in other genes involved in the biosynthesis of triterpenes. Analysis of the chemical phenotypes of these mutants, as well as morphological and physiological analyses, would be helpful in understanding the *in-planta* functions of these genes. The other is analysis of mutants that show resistance to inhibitors of triterpene biosynthesis. We recently reported the mutant *loi1*, which is resistant to lovastatin. The amount of sterols in *loi1* is less affected by lovastatin than that in the WT.[27)] The analysis of such chemical phenotypes is expected to be increasingly important in the future.

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