Functional Analysis of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Encoding Genes in Triterpene Saponin-Producing Ginseng1[C][W]

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Ginsenosides are glycosylated triterpenes that are considered to be important pharmaceutically active components of the ginseng (Panax ginseng ‘Meyer’) plant, which is known as an adaptogenic herb. However, the regulatory mechanism underlying the biosynthesis of triterpene saponin through the mevalonate pathway in ginseng remains unclear. In this study, we characterized the role of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) concerning ginsenoside biosynthesis. Through analysis of full-length complementary DNA, two forms of ginseng HMGR (PgHMGR1 and PgHMGR2) were identified as showing high sequence identity. The steady-state mRNA expression patterns of PgHMGR1 and PgHMGR2 are relatively low in seed, leaf, stem, and flower, but stronger in the petiole of seedling and root. The transcripts of PgHMGR1 were relatively constant in 3- and 6-year-old ginseng roots. However, PgHMGR2 was increased five times in the 6-year-old ginseng roots compared with the 3-year-old ginseng roots, which indicates that HMGRs have constant and specific roles in the accumulation of ginsenosides in roots. Competitive inhibition of HMGR by mevinolin caused a significant reduction of total ginsenosides in ginseng adventitious roots. Moreover, continuous dark exposure for 2 to 3 d increased the total ginsenosides content in 3-year-old ginseng after the dark-induced activity of PgHMGR1. These results suggest that PgHMGR1 is associated with the dark-dependent promotion of ginsenoside biosynthesis. We also observed that the PgHMGR1 can complement Arabidopsis (Arabidopsis thaliana) hmgr1-1 and that the overexpression of PgHMGR1 enhanced the production of sterols and triterpenes in Arabidopsis and ginseng. Overall, this finding suggests that ginseng HMGRs play a regulatory role in triterpene ginsenoside biosynthesis.

Ginseng (Panax ginseng ‘Meyer’), which belongs to the Araliaceae family, is a perennial herbaceous plant. It has been cultivated for over 2,000 years as a medicinal plant for its highly valued roots. The root of ginseng contains polyacetylenes, polysaccharides, peptidoglycans, phenolic compounds, and saponin (Kitagawa et al., 1987; Park, 1996; Radad et al., 2006). The triterpene saponins, referred to as ginsenosides, have been especially noted as active compounds contributing to the various efficacy of ginseng. Triterpenoid saponins are a class of secondary metabolites that are produced by a large number of plant species and predominantly found in dicot plants. They exhibit considerable structural diversity and notable biological activity (Hostettmann and Marston, 1995; Augustin et al., 2011). Ginsenosides are found exclusively in the plant genus Panax, with a content by dry weight of 4% (w/w) in the root (Shibata, 2001) and up to 6–10% (w/w) in the leaf, berry, and root hair (Shi et al., 2007). More than 150 naturally occurring ginsenosides have been isolated from Panax spp. (Shi et al., 2010). Among them, more than 40 ginsenosides have been isolated and identified from white and red ginseng from ginseng, showing different biological activities based on their structural differences (Gillis, 1997; Fuzzati, 2004; Xie et al., 2005; Li et al., 2009; Tung et al., 2009).

The main ginsenosides, constituting more than 80% of the total ginsenosides, are glycosides that contain an aglycone with a dammarenane skeleton (Fig. 1A). They include protopanaxadiol-type saponins (where sugar moieties are attached to the β-OH at C-3 and/or C-20), such as ginsenosides Re, Rg2, Rg3, and Rd, and protopanaxatriol-type saponins (where sugar moieties are attached to the α-OH at C-6 and/or the β-OH at C-20), such as ginsenosides Rb1, Rb2, Rc, and Re (Kim et al., 1987). The oleanane group has a pentacyclic

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structure, and only one ginsenoside, Ro, was identified, which is found in minor amounts in ginseng. These ginsenoside compounds contribute to the various pharmacological effects of ginseng, including antiaging (Cheng et al., 2005), antidiabetes (Attele et al., 2002), antiinflammatory (Wu et al., 1992), and anticancer activities, such as the inhibition of tumor-induced angiogenesis (Nakajima et al., 1998; Liu et al., 2000; Yue et al., 2007) and prevention of tumor invasion and metastasis (Sato et al., 1994; Mochizuki et al., 1995).

Ginsenosides are synthesized from the 30-carbon intermediate 2,3-oxidosqualene (a common precursor of sterols), which undergoes additional cyclization, hydroxylation, and glycosylation (Fig. 1B). Triterpene saponins, including ginsenosides, are derived from a universal precursor, isopentenyl diphosphate (IPP), which can be synthesized through the mevalonate (MVA) pathway in the cytosol (conserved in some prokaryotes and all eukaryotes) and the methylerythritol phosphate (MEP) in the plastids. The MVA pathway is controlled by the key regulatory enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMGR; EC 1.1.1.34; Bach, 1986). HMGR is known as a rate-limiting enzyme of the MVA isoprene pathway in plants and mammals (Goldstein and Brown, 1990). Because it is also known for rate-controlling cholesterol biosynthesis, its role in human health has been extensively studied. The inhibition of HMGR by statin has been applied as a major strategy for the treatment of cardiovascular disease and blood pressure reduction (Liao and Laufs, 2005). In contrast to the single HMGR in animals, plant HMGR is encoded by a multigene family, where the different isoforms exhibit spatial and temporal gene expression patterns. Two genes (named HMG1 and HMG2) in Arabidopsis (Arabidopsis thaliana) encode two HMGR isozymes with basically the same structural organization and intracellular localization, with one of them existing in a short form and one of them existing in a long form. However, the gene expression profiles of HMG1 and HMG2 are different (Lumbreras et al., 1995). The broad expression of HMG1 suggests that it may encode a housekeeping form of HMGR; correspondingly, the loss of function mutation of HMG1 caused senescence and sterility as well as a dwarf phenotype, all of which are likely related to the reduced sterol content (Suzuki et al., 2004). In contrast, hmg2 mutant did not display any distinct phenotype; hmg1 hmg2 double mutants are not viable because of the requirement of two genes for gametophyte development (Suzuki et al., 2009). Both HMG1 and HMG2 genes were also shown to play a major role in the

Figure 1. Biochemical pathway for the biosynthesis of ginseng saponins. A, Classification of main ginsenosides based on attached glycosides and the dammarendiol-type structure. Ara (fur), a-L-Arabinofuranosyl; Ara (pyr), a-L-glucopyranosyl; Glc, b-D-glucopyranosyl; Rha, a-L-rhamnopyranosyl. B, Ginsenoside biosynthesis pathway. b-AS, b-Amyrin synthase; CAS, cycloartenol synthase; DDS, dammarenediol synthase; FPS, farnesyl diphosphate synthase; GT, glucosyltransferase; Mev, a competitive inhibitor of HMGR; PPD, propanaxadiol type; PPT, propanaxatriol type; P450, cytochrome P450. Dotted line displays putative pathway. Reported enzymes in ginseng are shown with the National Center for Biotechnology Information accession numbers in parentheses. [See online article for color version of this figure.]
biosynthesis of triterpene metabolites (Ohyama et al., 2007). Although many reports reveal the pharmacological effects of ginsenosides and deal with the mass production of ginsenosides by genetic engineering and biotechnology, little is known about the regulatory mechanism of ginsenoside biosynthesis at molecular and biochemical levels in the ginseng plant. The lack of genome sequence information has led to several attempts to clone complete complementary DNA (cDNA) encoding enzymes involved in the postqualene step (indicated according to the accession number in Fig. 1B; Kushiro et al., 1998; Lee et al., 2004; Han et al., 2006, 2010, 2011, 2012; Kim et al., 2010). This study shows the isolation of the two full-length cDNA sequences together with the promoter sequences of HMGRs with structural and functional features in the ginseng plant. Phylogenetic analysis, the investigation of expression profiles of two ginseng HMGR genes (PgHMGRs), and the functional characterization of PgHMGR1 in genetic, molecular, and biochemical ways by heterologous overexpression in Arabidopsis and homologous ginseng plant were also carried out. Our findings indicate that PgHMGR1 is a functional ortholog of Arabidopsis HMGR (AtHMGR1), that both PgHMGRs are specifically tissue and age expressed, and that PgHMGR1 plays a regulatory role in the formation of triterpene ginsenosides in ginseng as well as other specialty plants (Singh et al., 2010; Alam and Abdin, 2011; Suwanmanee et al., 2013).

RESULTS

Isolation and Sequence Analysis of PgHMGR1 and PgHMGR2

To identify the first committed enzyme in the MVA pathway for the biosynthesis of isoprenoids, two EST clones coding PgHMGR (EC 1.1.1.34) were selected from previously constructed EST libraries from 14-year-old ginseng and hairy roots (Kim et al., 2006). Using RACE PCR, full-length cDNA sequences of PgHMGR1 and PgHMGR2 were obtained. PgHMGR1 has a length of 1,722 bp encoding 573 amino acids, whereas PgHMGR2 has a length of 1,785 bp encoding 594 amino acids (Supplemental Figs. S1 and S2). Moreover, a full genomic DNA sequence of each PgHMGR with the promoter sequence was obtained by genomic DNA walking. Both genes contain four exons and three introns (Supplemental Fig. S3A), which are typical features of the HMGR genes from other plant species. PgHMGR2 is 63 bp longer than PgHMGR1 in the first exon region, although the other three exons are the same length. The promoter region of PgHMGR1 contains G box (CACGTCG), whereas that of PgHMGR2 does not (Supplemental Figs. S1 and S2). PgHMGR1 and PgHMGR2 also show conserved domains (Supplemental Figs. S3B, S4, and S5), which are assumed to be characteristics of HMGR (Campos and Boronat, 1995). Both proteins contain a motif rich in arginines (RRR; Supplemental Figs. S1 and S2), which is predicted to be specific for their retention in the endoplasmic reticulum (ER; Schutze et al., 1994). The catalytic domains of PgHMGR1 and PgHMGR2 possess three catalytic active residues (EGC, DKK, and GQD) in the three conserved motif sequences predicted by Multi-ple EM for Motif Elicitation (Supplemental Figs. S3B and S4). This finding demonstrates that the features of HMGR are similar to the features of Cantharanthus roseus, which shows the active sites that are responsible for the binding of HMG-CoA and NADPH (Abdin et al., 2012).

PgHMGR1 and PgHMGR2 Are Predominantly Expressed in Roots

An age-dependent increase of ginsenosides has been reported in perennial ginseng root (Shi et al., 2007). To investigate the expression patterns of PgHMGR1 and PgHMGR2, quantitative reverse transcription (qRT) -PCR was performed using the ginseng seed, whole seedling, leaf, petiole, and root from a 2-week-old seedling and the flower, leaf, stem, main root, and lateral root of 3- and 6-year-old plants (Fig. 2A). A distinct anatomical feature of ginseng is the long petiole structure, with a relatively shortened stem in 2-week-old seedlings (Fig. 2A). The number of leaf petioles in the ginseng plant increases with the number of cultivation years, and the ginseng plant possesses five leaves at the age of 2 years. In the petioles of 2-week-old seedlings (Fig. 2B), PgHMGR1 was the most highly expressed, whereas PgHMGR2 was relatively weakly expressed. In 3-year-old ginseng (at which time most of the known ginseng organs are already formed) and 6-year-old ginseng, PgHMGR1 was expressed most abundantly in main roots and lateral roots (Fig. 2, C and D). Interestingly, PgHMGR1 expression was high in both 3- and 6-year-old ginseng at a significantly similar level (Fig. 2, C and D). PgHMGR2 was expressed at almost the same level as PgHMGR1 in the roots of seedlings, and its expression gradually increased as it aged (Fig. 2, E to G). These results suggest that PgHMGR1 and PgHMGR2 are necessary for organ development, especially in roots. PgHMGR1 may also play a role in general metabolites production, whereas PgHMGR2 may play a role in age-dependent specific metabolites production in roots. When the promoter sequence of PgHMGR1 (~1,317 to 1 bp), which was fused with GUS (pHMGR1::GUS), was transformed into a ginseng adventitious root, it was highly expressed in the whole root, with more restriction in the vasculature and root tip (Fig. 2I). When pHMGR1::GUS and pHMGR2::GUS were expressed in Arabidopsis, expressions were also observed in the whole-root vasculature (Fig. 2, Hb and Ib) and an 8-d-old main root tip (Fig. 2, Hc and Ic), similar to the ginseng plant. This finding suggests that Arabidopsis is an ideal plant for additional functional characterization of PgHMGR. The precise expression of pHMGRs::GUS in the overall tissues of a germinating...
embryo (Fig. 2, Ha and Ia), cotyledon, true leaf, and root was also observed in Arabidopsis (Fig. 2, Hd and Id). GUS expression was high in the filament, flower sepal, and pistil style (Fig. 2, He and Ie), as well as the junction between the silique and pedicel of a 50-d-old plant (Fig. 2, Hf and If).
Activity of HMGR Is Positively Correlated with Ginsenoside Production

To understand whether HMGR activity is involved in ginsenoside biosynthesis, the inhibition of HMGR activity by mevinolin (Mev) was conducted. Mev (6a-methylcompactin), also referred to as lovastatin, competitively inhibits the binding of the HMG-CoA substrate to the active site of the HMGR enzyme and consequently, blocks the synthesis of cytosolic IPP (Bach and Lichtenthaler, 1982) and phytosterol biosynthesis (Bach and Lichtenthaler, 1987; Bach et al., 1990). Mev treatment for 1 d in 4-week-old adventitious roots significantly decreased the total contents of major ginsenosides by about 34% compared with the control plants (Fig. 3A; Supplemental Fig. S6). This result shows that the ginsenosides are rapidly turned over. Transcripts of *PgHMGR1* and *PgHMGR2* were initially down-regulated within 1 d, and *PgHMGR1* started to become stabilized (Fig. 3B). This finding indicates that *PgHMGR2* follows an independent regulatory pathway, possibly through posttranscriptional regulation. However, it is observed that the metabolic fluctuation of ginsenoside is more correlated with HMGR activity (Fig. 3C). Additionally, methyl jasmonate (MJ), known as an elicitor of triterpene biosynthesis, increased the major ginsenoside contents as well as the transcripts of *PgHMGR1* in ginseng adventitious root (Fig. 3, D and E; Supplemental Fig. S6). The mismatching of the ginsenosides contents with the transcript levels of the HMGR genes and the enzyme activity of HMGR might be explained by the specific posttranscriptional and feedback regulations of HMGR. The promoter::GUS fusion of *PgHMGR1* also supports the observed results (Fig. 3, B, E, and F), and these results corroborate a role of HMGRs in ginsenoside biosynthesis.

*PgHMGR1* Is Dark Dependent and Possibly Associated with Ginseng Shadowing Growth

The dark-dependent hypocotyl expression pattern of *pHMGR1::GUS* suggested the hypothesis that...
ginsenoside biosynthesis might be regulated by dark treatment. Placing ginseng plants under completely dark conditions for 48 h caused a dramatic increase of ginsenoside contents compared with the controls grown under a 16-h-light/8-h-dark condition (Fig. 4, A and B). Dark conditions for 72 h also resulted in an increase of ginsenosides at a relatively weak level compared with that for 48 h. However, the HMGR activity gradually increased in both the leaves and roots for up to 3 d (Fig. 4C). This result indicates that, up to a certain threshold level, the activity of HMGRs positively up-regulates the ginsenosides contents. It is also considered that the contents of several individual ginsenosides show independent modulation, where Rb1 in the leaf and Rg2 in the root are not significantly changed (Supplemental Fig. S7). Transcripts of PgHMGR1 and PgHMGR2 were significantly decreased in the ginseng leaf and root in dark conditions (Fig. 4, D and E). The behavior of PgHMGR2 in the root somewhat differs from that of PgHMGR1, and this result is similar to that seen in the adventitious roots (Fig. 3). PgHMGR2 seems to follow a different posttranscriptional regulation pathway in roots but did not play a major role in altering the final production of ginsenosides (Figs. 3 and 4). The promoter sequence of PgHMGR1 contains a G box, which is known as a putative phytochrome-interacting factor3 (PIF3) binding motif (Fig. 4F). Thus, the precise expression of pHMGR1::GUS was analyzed further in different developmental stages. The activity pHMGR1::GUS was
differentially modulated by light exposure in the leaf and hypocotyl (Fig. 4G). GUS expression was induced in the hypocotyl of a 4-d-etiolated seedling and reversed by light exposure within 2 h. However, its expression after dark treatment decreased in the 7-d-old seedling, where true leaves are emerged (Fig. 4H). These results show that the transcripts of \( \text{PgHMGRs} \) are dark-dependently regulated in different developmental stages and ultimately, affect the ginsenoside production.

**Subcellular Localizations of \( \text{PgHMGR1} \) in Arabidopsis**

Fractionation analysis identified that plant HMGR is located in three subcellular sites: plastids, mitochondria, and ER (Brooker and Russell, 1975; Wilson and Russell, 1992). Despite the observed enzymatic activity in several organelles, immunofluorescence confocal microscopy and immunogold electron microscopy analyses showed that \( \text{AtHMGR1} \) is localized into the ER and in the unidentified spherical vesicles that were not colocalized with peroxisomal catalase (Leivar et al., 2005). Cyan fluorescent protein (CFP) or monomeric red fluorescent protein (mRFP) was tagged in the C-terminal ends of \( \text{PgHMGR1} \) and \( \text{PgHMGR2} \) to verify subcellular localization patterns by confocal laser scanning microscopy. Both \( \text{PgHMGR1} \) and \( \text{PgHMGR2} \) were localized in the intracellular vesicles (Fig. 5, A to C). To verify the spherical vesicles and other unidentified vesicles, mRFP-tagged \( \text{PgHMGR1} \) lines were crossed with ER (ER-yk), peroxisome (PX-yk), and plastid (PT-yk) markers; all markers were tagged with yellow fluorescent protein (YFP). \( \text{PgHMGR1} \) was completely comerged with ER and plastid as well as partially comerged with peroxisome (PX; Fig. 5E). The catalytic domain of \( \text{PgHMGR1} \) was localized in the cytosol (Fig. 5D), similar to the case of Arabidopsis HMGR1 (Leivar et al., 2005).

**Overexpression of \( \text{PgHMGR1} \) Enhances Production of Triterpenes in Arabidopsis and Ginseng**

To investigate whether increased HMGR activity contributes to metabolite profiles, the sterol contents of overexpression lines (\( \text{HMGR1ox} \)) in Arabidopsis were analyzed using gas chromatography-mass spectrometry (GC-MS) analysis. Higher plants synthesize a mixture of phytosterols, including campesterol, stigmasterol, and \( \beta \)-sitosterol (Hartmann and Benveniste, 1987). Pentacyclic \( \beta \)-amyrin, one of the most common triterpenes in plants, and \( \alpha \)-amyrin are present in Arabidopsis. Therefore, these two triterpenes and the three major phytosterols were analyzed together with squalene, a common precursor of triterpene and sterol. Total sterols were extracted from the rosette leaves and inflorescence of 5-week-old plants. Compared with the wild-type control, the rosette leaves of \( \text{HMGR1ox} \) (no. 15-8) showed 2 times higher \( \beta \)-sitosterol, 1.8 times higher campesterol and cycloartenol, 2.5 times higher \( \beta \)-amyrin, and 2 times higher \( \alpha \)-amyrin contents (Fig. 6A). In inflorescence, it showed 1.6 times higher campesterol, \( \beta \)-sitosterol, and \( \beta \)-amyrin, 2.6 times higher cycloartenol, and 3.7 times higher \( \alpha \)-amyrin (Fig. 6B) than the wild-type control. The contents of squalene and stigmasterol were not significantly changed compared with the contents of the control. To verify direct evidence of HMGR on ginsenoside biosynthesis, \( \text{PgHMGR1} \) was constitutively overexpressed under 35S promoter in

**Figure 5.** Subcellular localization of \( \text{PgHMGR1} \), \( \text{PgHMGR2} \), and the catalytic domain of \( \text{PgHMGR1} \). A and B, Fluorescent images of \( \text{PgHMGR1-CFP} \) and \( \text{PgHMGR1-mRFP} \) were visualized by a confocal laser scanning microscope. C, A fluorescent image of \( \text{PgHMGR2-CFP} \) was visualized with green color. D, The catalytic domain of \( \text{PgHMGR1} \) tagged with CFP at the C terminus was localized in the cytosol. E, Fluorescent signals of \( \text{PgHMGR1-mRFP} \) merged with ER-yk, PX-yk, and PT-yk. Bars = 5 \( \mu \text{m} \). [See online article for color version of this figure.]
the ginseng adventitious roots that were derived from transgenic ginseng calli. The amount of individual ginsenosides in the four different transgenic lines was 1.5 to 2 times greater than the amount in the control without altering the ratio of individual ginsenoside (Fig. 6C).

Conserved Role of \(\text{PgHMGR}_1\)

\(\text{PgHMGR}_1\) and \(\text{PgHMGR}_2\) showed 74.8% and 67.8% of amino acid sequence identity with \(\text{AtHMGR}_1\) (At1g76490) and \(\text{AtHMGR}_2\) (At2g17370), respectively (Enjuto et al., 1994). \(\text{PgHMGR}\) is clustered into plant HMGRs and structurally distinct from fungus and animal enzymes (Supplemental Fig. S8A). Three conserved motif sequences were identified in both plant and animal HMGRs (Supplemental Figs. S5 and S8B). The conserved role of ginseng HMGRs was revealed by the genetic complementation of Arabidopsis null mutant \(hmgr_1-1\) (Fig. 7, A, B, and D). The homozygous \(hmgr_1-1\) showed a severe dwarf phenotype with partial restoration by squalene supply (Fig. 7C). The overexpression of the full-length cDNA sequence of \(\text{PgHMGR}_1\) and the catalytic domain of \(\text{PgHMGR}_1\) under the 35S promoter complemented the phenotypic defect of \(hmgr_1-1\), whereas that of \(\text{PgHMGR}_2\) did not, indicating that \(\text{PgHMGR}_1\) is a functional ortholog of \(\text{AtHMGR}_1\) (Fig. 7, C and E to G; Supplemental Table S1).

DISCUSSION

In plants, triterpenoids and sesquiterpenoids are biosynthesized through the MVA pathway, whereas monoterpenoids, diterpenoids, and tetraterpenoids are biosynthesized through the MEP pathway. The formation of MVA is catalyzed by HMGR (Bach and Lichtenthaler, 1982), and it serves as the common precursor for the production of a number of natural products. Here, we report the whole-genome structure of two ginseng HMGR genes encompassing the promoter sequence, the subcellular localization of corresponding HMGR enzymes, and the importance of dark-regulated HMGR activity for the production of ginsenosides and sterols. In this study, ginseng plants and ginseng adventitious roots overexpressing \(\text{PgHMGR}\) as well as Arabidopsis plants heterologously overexpressing the same gene were used for the functional characterization of \(\text{PgHMGR}\) at genetic and biochemical levels (Fig. 8).

Tissue-Specific Expression Patterns and Developmental Roles of \(\text{PgHMGRs}\)

In contrast to the single HMGR in animals, archaea, and eubacteria, HMGR in plants is encoded by multiple HMGR genes that seem to have arisen by gene duplication and subsequent sequence divergence. In the ginseng plant, two isoforms of HMGR were identified and showed differential expression patterns with conserved structural divergences (Fig. 2; Supplemental Fig. S3) in ginseng. Although one HMGR sequence of \(\text{Panax quinquefolius}\) (ACV65036) was reported in the National Center for Biotechnology Information to have 76% identity with the amino acid sequence of \(\text{PgHMGR}_1\), a functional study of HMGR has not yet been performed in ginseng. The transcripts of \(\text{PgHMGR}_1\) were found to be predominant in the petioles of the seedling stage and were enhanced in the roots of the 3- and 6-year-old
ginseng. However, the highest expression of *PgHMGR1* in the root was found at almost the same level in ginseng from the seedling stage to 3- and 6-year-old plants, whereas the transcripts of *PgHMGR2* in the root were gradually increased with age (Fig. 2, B to G). It suggests that *PgHMGR1* plays a major role in providing sterols or triterpenes in ginseng roots, whereas *PgHMGR2* might have a more specific role in the age-dependent production of certain metabolites. Sterol biosynthesis-related genes were intended to be expressed constitutively in all plant tissues to synthesize sterol, which is necessary for plant development (He et al., 2003; Shen et al., 2006). A detailed functional understanding of sterol and triterpene in plant development needs to be further clarified. Corresponding to the expression of *PgHMGR1* and *PgHMGR2* in the vasculature tissue of the roots (Fig. 2, Hb and Ib), the ginsenoside biosynthesis-related genes *Pg squalene synthases* (SSs) and *Pg squalene epoxidases* (SEs) were also characterized as being expressed in vascular bundle tissues, including phloem cells, parenchymal cells near the xylem, and resin ducts in the petioles of ginseng (Han et al., 2010; Kim et al., 2011). It can be postulated that the phloem and resin ducts might serve as metabolically active sites for sterol and saponin biosynthesis and play a role in the production of squalene oil. In fact, ginsenoside is accumulated to a greater degree in the phloem than the xylem (Fukuda et al., 2006) and is observed in parenchymal and phloem cells (Yokota et al., 2011).

The expressions of *pHMGR1::GUS* and *pHMGR2::GUS* were detected at the early postgermination stage in ginseng seedlings and also triggered during the germination stage in Arabidopsis. These results suggest that some triterpene is required for the germination stage in the seedling, which is reminiscent of the active β-amyrin production in pea seedlings (Baisted, 1971). Interestingly, *PgHMGR1* was highly expressed in petiole tissue (Fig. 2B), similar to the high expression pattern of other ginsenoside biosynthesis-related genes encoding SS (Kim et al., 2011) and SE genes (Han et al., 2010). A long petiole is a distinct anatomic structure that can also be found in ginseng. The relatively high expression of *PgHMGR1* in petiole may function on cell growth by producing sterols that are known as essential constituents of membranes of eukaryotic cells and/or may function on plant defense by producing derivative triterpenes. The promoter activity of *PgHMGR1* and *PgHMGR2* was high in the early flowering stage, with no detection in matured silique or seed. High HMGR expression in the early developmental stage has also been shown in other plants: potato (*Solanum tuberosum*) *HMGR1* is expressed in the early flower developmental stage (Korth et al., 1997), and tomato (*Solanum lycopersicum*) *HMGR1* is expressed in young tomato fruit (Julesko et al., 1999). The flower phenotype in tobacco (*Nicotiana tabacum*) is altered by the overexpression of *AtHMGR1* (Hey et al., 2006), which suggests that HMGR-derived phytosterols and metabolites play roles in flower development (Fig. 6B). It has also been reported that the constitutive inhibition of HMGR by Mev ultimately blocks fruit development in the early stages (Narita and Gruissem, 1989).
PgHMGR1 Contributes to Ginsenoside and Sterol Overproduction

Unfortunately, ginseng is not practically amenable to loss-of-gene or gain-of-gene function studies. To gain an insight into the effects of the loss of HMGR activity on ginsenoside biosynthesis, an inhibitor was used to deplete the metabolic flux through the MVA pathway. Treatment with Mev, a highly potent competitive inhibitor of HMGR, reduced the total ginsenoside content drastically in adventitious roots compared with the control after reduced gene expression, which is comparable with the well-known elicitor effect by MJ (Fig. 3). In adventitious roots, the increment of MJ-induced ginsenosides is almost correlated with the expression level of PgHMGR1, whereas PgHMGR2 seems to be working independently or rather, inhibitory to the activity of PgHMGR1 in a certain stage. Here, the possible posttranscriptional modification also needs to be considered, and it will explain the gaps between expression and activity for the ginsenoside production. The apparent Mev-induced HMGR activity (Fig. 3C) can be explained by the increased translation or decreased degradation of the proteins through the feedback regulatory mechanism (Hemmerlin et al., 2003). Importantly, overexpressing PgHMGR1 in ginseng adventitious roots actually resulted in increased ginsenosides contents (Fig. 6C).

In addition, to test whether PgHMGR1 can contribute to the triterpene pathway in other plants, PgHMGR1 was further expressed in Arabidopsis. Although HMGR is known as the key regulatory enzyme of sterol biosynthesis in plants (Babiychuk et al., 2008), overexpression of HMGR in Arabidopsis did not show any altered morphology or isoprenoid content (Re et al., 1995). However, overexpression of the Hevea brasiliensis HMGR in tobacco increased the amount of sterol and mainly, the intermediates of the sterol pathway in the form of fatty acyl esters in accordance with the increased activity of HMGR (Chappell, 1995; Schaller et al., 1995). Sterol-enriched tomato mutant also showed greater accumulation of biosynthetic intermediates that were esterified with fatty acids and stored in cytoplasmic lipid droplets (Maillot-Vernier et al., 1991). In the case of the overexpression in tomato, phytosterols were elevated without altering the activity of HMGR (Enfissi et al., 2005). The constitutive overexpression of PgHMGR1 driven by the 35S promoter resulted in the accumulation of not only sterols but also, triterpenes in Arabidopsis (Fig. 6, A and B), with a modest increase of HMGR activity. Based on the previous reports, it should also be noted that the major type of sterols in PgHMGR1ox lines are esterified.

Tight Regulation of HMGR Activity by Dark Contributes to Ginsenoside Biosynthesis

Plant HMGR is modulated by myriad cellular and environmental signals, such as plant hormones, calcium, chemical challenge, pathogen, wounding, and especially, light (Stermer et al., 1994). The dark-induced expression of HMGR1 promoter activity in Arabidopsis immature leaves and seedlings (Learned and Connolly, 1997) as well as increased HMGR activity in etiolated pea seedlings (Brooker and Russell, 1979) are in direct contrast to the suppression of HMGR activity in dark-treated mature green potato plants (Korth et al., 2000). In the case of ginseng HMGR, the dark treatment induced the promoter activity of PgHMGR in etiolated hypocotyl (Fig. 4G), which was reversed in the true leaves of pHMGR1::GUS Arabidopsis (Fig. 4H). Reduced transcripts of PgHMGR1 and PgHMGR2 were also observed in the ginseng plant (Fig. 4D). The differential regulation of the pHMGR1::GUS expression in leaf and hypocotyl tissue can be explained by an organ-autonomous response (Learned and Connolly, 1997). Down-regulation of HMGR in Arabidopsis because of light was shown to occur through photoreceptor phytochrome B and transcription factor LONG HYPOCOTYL5 (HY5; Rodríguez-Concepción et al., 2004). Analysis of the promoter sequence implied that PgHMGR1 might be regulated by PIF3 binding to the G box in the region of PgHMGR1 promoter (Fig. 4F), which requires further confirmation by experimental analysis. PIFs, basic helix-loop-helix transcription factors, play a central role in the light-mediated responses (Castillon et al., 2007). Shin et al. (2007) showed that PIF3 and HY5 regulate the anthocyanin biosynthesis by binding to the promoters of anthocyanin biosynthesis-related genes. PIF3 is also known as a negative component in the phytochrome B-mediated inhibition of hypocotyl elongation (Kim et al., 2003). Taken together, it suggests that ginsenoside biosynthesis is...
tighty regulated by light, possibly through PIF3-mediated regulation. When the whole ginseng plant was kept in the dark for up to 48 h, the ginsenoside content increased 24% in leaf and 35% in root compared with the control (Fig. 4, A and B). This result might also be because of the dark stress by the light-to-dark switch (Souret et al., 2003). The contents of naturally synthesizing ginsenosides and the ratio of protopanaxatriol-protopanaxadiol types of ginsenosides are different between species and organs (Wang et al., 1999; Qu et al., 2009). The differential regulation of PgHMGR genes in leaf and root by the dark can be a good starting point to explain the differential ginsenoside biosynthesis. In mature ginseng leaf, both PgHMGR genes are coordinately down-regulated (Fig. 4D). However, the gene expression of PgHMGR2 in root behaves in different ways in initial stages of the treatment (Figs. 3B and 4E). In 3- and 6-year-old roots, where the expression patterns of PgHMGR1 are constant and PgHMGR2 is differentially up-regulated with perfect age matches with these differential transcriptional regulations (Fig. 2). Thus, we speculate that PgHMGR1 plays general housekeeping roles, whereas PgHMGR2 plays stress- and time-dependent specific roles, which still need to be clarified. However, dark-induced HMGR activity in the ginseng plant for 2 to 3 d was positively correlated with the total ginsenoside content (Fig. 4, A and C).

PgHMGR1 Localized into the Cytoplasmic Organelles and Its Gene Complements AtHMGR1

Both PgHMGR1 and PgHMGR2 contain an RRR motif at their N terminus, which may act as an ER retention signal (Schütze et al., 1994; Merret et al., 2007). When PgHMGR1 and PgHMGR2 were expressed individually in Arabidopsis as C-terminal CFP fusions, both proteins were also targeted to the ER and vesicle-like structures and merged with the ER, PX, and plastid markers (Nelson et al., 2007; Fig. 5). It suggests that ginseng HMGR could be targeted to different subcellular organelles through posttranslational modifications. We cannot exclude the possibility of alternative mRNA processing, which might contribute to a differential localization. For example, the long isoform of IPP isomerase was targeted to the chloroplast and mitochondria, whereas its short isoform was localized to the peroxisome (Sapir-Mir et al., 2008). The catalytic domain of ginseng HMGR was localized to the cytosol, confirming that the N-terminal domain of PgHMGR is necessary for binding to the membrane, supporting a previous report (Leivar et al., 2005). In mammals, the peroxisomal localization of HMGR in rat (Keller et al., 1985) was reported in addition to ER localization (Goldfarb, 1972); thus, dual localization of HMGR in the PX and ER needs to be considered (Kovacs et al., 2007). The localization of PgHMGR1 in multiple subcellular compartments may play roles in the specialized localization of ginsenoside Rb1 in the cytosol, plastid, and PX in the ginseng plant (Yokota et al., 2011). The plastidial MEP pathway can also contribute to the localization of ginsenosides in distinct compartments. Here, the possible cross talk between the MVA and MEP pathways may also be involved (Hemmerlin et al., 2003).

Although PgHMGR1 was expressed in different levels among the tested organs, the PgHMGR1 gene seems to be expressed constitutively throughout ginseng development, which was similar to the characterized homologs from Salvia miltiorrhiza (Liao et al., 2009) and Eucommia ulmoides (Jiang et al., 2006). Indeed, the heterologous expression of the two full-length ginseng HMGRs in the Arabidopsis hmgr1-1 mutant background showed that only PgHMGR1 could complement the dwarf and sterile phenotypes of hmgr1-1 (Suzuki et al., 2004), which suggests that PgHMGR1 is a functional ortholog of AtHMGR1. The defect of AtHMGR1 could also be complemented by the catalytic domain of PgHMGR1, suggesting that the product of PgHMGR1 is sufficient to complement without proper targeting to an organelle. Although PgHMGR1 shares more identity with AtHMGR1 than with AtHMGR2, it has the closest relationship with EsHMGR, HMGR in Eleutherococcus senticosus, which is a small and woody shrub of the same family known to have similar herbal properties as ginseng (Huang et al., 2011). PgHMGR2 shares a high sequence identity with HMG2 (CaHMGR and AAB69727) from Campylothece acuminata (Maldonado-Mendoza et al., 1997), which also showed lower expression than HMG1. This result suggests that PgHMGR1 and PgHMGR2 originated from a gene evolution event after the split from Arabidopsis and before the separation of PgHMGR1 and its homologs. Considering these findings, it is possible that each of the isozymes of HMGR could have evolved separately according to the production of specific products rather than by duplication.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Columbia ecotype (CS6000) of Arabidopsis (Arabidopsis thaliana) was used as a model plant in this study. ER-YPF-expressing lines (ER-yk; CS16251), PX-YPF-expressing lines (PX-yk; CS16261), MT-YPF-expressing lines (MT-yk; CS16264), and PT-YPF-expressing lines (PT-yk; CS16267; Nelson et al., 2007) and hmgr1-1 (SALK_125439) were purchased from the Arabidopsis Stock Center (http://www.Arabidopsis.org/). Seeds were surface sterilized and sown on one-half-strength Murashige and Skoog (Duchefa Biocheme) containing 1% (w/v) Suc, 0.5 g/L 2-[N-morpholino]ethanesulfonic acid (pH 5.7) with KOH, and 0.8% (w/v) agar. Three-day cold-treated seeds were germinated under a long-day condition of 16 h of light and 8 h of dark at 23°C. The Korean ginseng (Panax ginseng ‘Yunpoong’) seeds used in this study were obtained from the Ginseng Bank in South Korea. Transformants were selected on hygromycin-containing plates (50 μg/ml). Ten-day-old seedlings were transplanted into soil and allowed to grow for up to 5 weeks under the same light and dark conditions. For RNA extraction, seedlings were grown on plates for 15 d. For metabolite analysis, leaves and the inflorescence from 5-week-old plants were collected.

Ginseng Materials and Treatment

Mev and MJ were purchased from Sigma. Stock solutions of Mev (10 μM) and MJ (100 μM) in ethanol were stored at −20°C. For inhibitor treatment of
ginseng adventitious roots, after 4 weeks of precultivation, 10 μM Mev or 10 μM MJ was added. After 1, 3, and 7 days, harvested adventitious roots were frozen and used for RNA extraction, HMG activity assay, and ginsenoside analysis. Three-year-old ginseng plants were hydroponically grown in perlite and peat moss at 23°C ± 2°C under white fluorescent light (60–100 μmol m⁻² s⁻¹) in a controlled greenhouse (provided by i-farm, Yeo-Ju, Korea) and used for dark treatment. Control plants were grown in 16 h of light and 8 h of dark and sampled in light conditions, whereas dark treatments (covered with a black box) lasted for 2 and 3 d. Leaf and root parts were separately used for RNA isolation, HMG activity assay, and ginsenoside analysis.

Identification of PgHMGR Genes and Sequence Analysis

To obtain a full-length coding sequence of the PgHMGR gene, RACE PCR was performed using a Capfishing full-length cDNA premix kit (Seegene) and HotStarTag (Qiagen) as a DNA polymerase. The first strand cDNA synthetic reaction from total RNA was catalyzed by superscript III RNase H reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s instructions, and the cDNA was prepared using a commercial cDNA synthesis kit (Clontech). Specific primers were designed according to the 3′- and 5′-end sequences of partial PgHMGR ESTs, which were derived from our ginseng EST library. The primer sequences used were as follows: for HMGR1, HMG1,2,5′-ATG GAG GCC ATT AAC GAT GAA AAA G-3′; HMG1,2,5′-ACC AAC CTC AAT TGA TGG CAT GTG G-3′; HMG3,5′-CTT TCT TCG CTG AGA AAA CCT CAC CA-3; and for HMGR2, HMG2,5′-CTC TCA CTC GAG TAC AAA CTT GGC GAC-3′; HMG2,1,5′-GAG ATG CTA ATT GGG TCA CCC AAC ACC-3′; HMG2,3,5′-ATT GCC TCA CAA ACA ACC GAT TTA CC-3′. RACE PCR was performed by the hot-start method with the following conditions: 30 cycles at 94°C for 40 s, 60 cycles at 66°C for 40 s, 72°C for 80 s, and a final extension of 72°C for 5 min. The PCR product was purified and ligated into a pGEM-T vector (Promega) followed by sequence. By assembling the sequence of the 5′-RACE and 5′-RACE products, the full-length cDNA sequence of PgHMGRs was deduced. Sixty nanograms of genomic DNA of cv Yunpoong and a pair of PCR primers (start codon and stop codon) were used for amplifying the genomic sequence of PgHMGR. Dedenied amino acid sequences were searched for homologous proteins using the BLASTX databases. ClustalX with default gap penalties was used to perform multiple alignments. A phylogenetic tree was constructed by the neighbor-joining method, and the reliability of each node was established by bootstrap methods using MEGA4 software. Identification of the conserved motifs of HMGR was accomplished with Multiple EM for Motif Elcitation.

Isolation of Promoter Sequences

The Universal Genome Walker Kit (Clontech Laboratories, Inc.) was used to isolate fragments of the PgHMGR1 and PgHMGR2 promoter. Ten 6-bp recognizing and blunt end-forming restriction enzymes (DraI, EcoRV, PstI, Stul, Sphi, Small, Mscl, Scal, EcoISI, and Hpal) were used to digest the isolated cv Yunpoong genomic DNA. DNA fragments containing the adaptors at both ends were used as templates for amplifying the PgHMGR promoter regions. The first PCR, using adaptor primer (API) and gene-specific primer (GSP) (H1-GSP1, 5′-ATA ACA GTC CCC GAG TTT TAC CAG CAC-3′; H2-GSP1, 5′-GCT TGG GAG GAA GAC AGT CAT TAG C-3′), and the second nested PCR, using API and H1-GSP2 and H2-GSP2 (H1-GSP2, 5′-AGC TGA GAC AAA TTA TGG GAC GAA ATC-3′; H2-GSP2, 5′-ATG CTC GAC GAC TAT CCT CCT GCT T-3′), were performed using i-MAXII (Intron). The amplified fragment was cloned in the pGEM-T vector and sequenced. The promoters were analyzed using PlantPan (Plant Promoter Analysis Navigator).

Vector Construction and Arabidopsis Transformation

To visualize the subcellular localization patterns of ginseng HMGs, cDNA sequences of PgHMGR1 and PgHMGR2 were cloned into a pCAMBIA1390 vector containing the Cauliflower Mosaic Virus 35S promoter and enhanced CFP (eCFP). The PgHMGR1 cDNA and its catalytic domain (PgHMG1C1D; amino acid residues 154–573) were amplified using primers with Xhol and EcoRI sites (underlined) for PgHMGR1, 5′-CA TCT CAC CAA TAT GCC TGA TCT ATG TGC TAC GAC GAG-3′ and 5′-TG GAA TTC ACA TCC AAA AAT TTG GGA GAC-3′; PgHMG1C2D, 5′-TAA CTC GAC ATG CCC ATA GTC AGT TGC TAC-3′ and 5′-TG GAA TTC ACA TCC AAA AAT TTG GGA GAC-3′; and used for dark treatment. Control plants were grown in 16 h of light and 8 h of dark and sampled in light conditions, whereas dark treatments (covered with a black box) lasted for 2 and 3 d. Leaf and root parts were separately used for RNA isolation, HMG activity assay, and ginsenoside analysis.
close to zero, indicating that the efficiencies of the gene and the internal control β-actin were equal.

Confocal Microscopy Analysis

The fluorescence from reporter proteins and organelle markers was observed by confocal laser scanning microscopy (LSM 510 META; Carl Zeiss). GFP, CFP, YFP, and mRFP were detected using 488–505 to 530-, 458–475- to 525-, 514–>530-, and 543–560- to 615-nm excitation/emission filter sets, respectively. Fluorescence images were digitized with the Zeiss LSM image browser.

HMGR Activity Assay

Extraction of crude proteins was performed as described (Rodriguez-Conception et al., 2004), with minor modifications. Two-week-old seedlings of Arabidopsis, leaves or roots of 3-year-old ginseng, or adventitious roots of ginseng (approximately 200 mg) were homogenized in liquid nitrogen and mixed with 1 mL of prechilled extraction buffer containing 100 mM Suc, 40 mM sodium phosphate (pH 7.5), 30 mM EDTA, 50 mM NaCl, 10 mM dithiothreitol, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 μM bestatin, 15 μM E64, 20 μM leupeptin, 15 μM pepstatin, 0.5 mM chaulmoogra, 0.5 mM phenylmethylsulfonyl fluoride, and 0.25% (w/v) Triton X-100. The slurry was centrifuged at 200°C for 4 min to remove cell debris, and the supernatant was then used for the determination of HMGR activity as previously described (Dale et al., 1995) with the following modifications. The protein concentration was determined with the BCA Protein Assay Kit (Intron). The supernatant was analyzed using a spectrophotometric assay at 37°C (total volume of 200 μL) containing 0.3 mM HMG-CoA, 0.2 mM NADPH, and 4 mM dithiothreitol in 50 mM Tris-HCl (pH 7.0). The decrease in A340 was monitored at 37°C. One unit of HMGR activity is defined as the amount of enzyme that oxidizes 1 μmol NADPH min⁻¹ at 37°C.

GUS Histochemical Analysis

Four-day-old seedlings were treated with the indicated chemical for 3 h before visualizing the GUS activity. GUS staining was performed by incubating whole seedlings in the staining buffer containing 1 mM 3-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (Duchefa Biochem), 0.1 M NaH2PO4, 0.1% (w/v) Triton-X, and 0.5 mM potassium ferricyanide and ferrocyanide at 37°C until a blue color appeared (1–3 h). Stained seedlings were cleared in 70% (v/v) ethanol and then 100% (v/v) ethanol for 2 h each. In the final step of dehydration, samples were sequentially exposed to 10% (v/v) glycerol/50% (v/v) ethanol and then 100% (v/v) ethanol for 2 h each. In the final step of dehydration, samples were sequentially exposed to 10% (v/v) glycerol/50% (v/v) ethanol and then 100% (v/v) ethanol for 2 h each. In the final step of dehydration, samples were sequentially exposed to 10% (v/v) glycerol/50% (v/v) ethanol and then 100% (v/v) ethanol for 2 h each.

HPLC Analysis of Ginsenosides Extracted from Ginseng

For the analysis of ginsenosides from different ginseng samples, 0.3 to 1 g of milled powder of freeze-dried adventitious roots, leaves, and roots was soaked in 80% (v/v) MeOH at 70°C. After evaporation, the residue was dissolved in water, followed by extraction with water-saturated n-butanol. The butanol layer was then evaporated to produce the saponin fraction. Each sample was dissolved in MeOH (1 g/5 mL) filtered through a 0.45-μm filter, and used for HPLC analysis. The HPLC separation was carried out on an Agilent 1260 series HPLC system. This experiment used a C18 (250 × 4.6 mm, i.d. = 5 μm) column using acetonitrile (solvent A) and distilled water (solvent B) mobile phases, with a flow rate of 1.6 mL/min and the following gradient: A:B ratios of 80:20 for 0 to 29 min, 70:30 for 29 to 36 min, 68:32 for 36 to 45 min, 66:34 for 45 to 47 min, 64:35:55 for 47 to 49 min, 0:100 for 49 to 61 min, and 80:20 for 61 to 66 min. The sample was detected by UV spectrometry at a wavelength of 203 nm. Quantitative analysis was performed with a one-point curve method using external standards of authentic ginsenosides.

GC-MS Analysis of Sterols and Triterpenes Extracted from Arabidopsis

Using the method modified from Suzuki et al. (2004), freeze-dried plant materials (rosette leaves, 200 mg; inflorescence, 25 mg) were powdered and then extracted two times with 1 mL of CHCl₃:MeOH (7:3) at room temperature. 5-α-Cholestane (20 μg) was used as an internal standard. The extract was dried in a rotary evaporator and saponified with 1.5 mL of MeOH and 20% (v/v) KOH aqueous for 1 h at 80°C to hydrolyze the sterol esters. After saponification, 1.5 mL each of MeOH and 4 N HCl were added for 1 h at 80°C, and these reaction mixtures were extracted three times with 4 mL of hexane. When the phases separated, the sterols partitioned to the hexane layer, and the combined hexane layer was evaporated to dryness. The residue was trimethylsilylated with pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (1:1) at 37°C for 90 min and then analyzed by capillary GC-MS. GC-MS analysis was performed using a mass spectrometer (HP 5973 MSD) connected to a gas chromatograph (6890A; Agilent Technologies) with a DB-5 (MS) capillary column (30 m × 0.25 mm, 0.25-μm film thickness). The analytical conditions were as follows: electron ionization, 70 eV; source temperature, 250°C; injection temperature, 250°C; column temperature program of 80°C for 1 min, then increased to 280°C at a rate of 10°C/min and held at this temperature for 17 min; posttemperature, 300°C; carrier gas, He; flow rate, 1 mL/min; run time, 38 min; and splitless injection. The endogenous sterol levels were determined as the peak area ratios of molecular ions of the endogenous sterol and internal standard. Standards, including squalene, phytosterols (campesterol, β-sitosterol, and stigmasterol), and triterpene (δ-amyrin and α-amyrin) were purchased from Sigma.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. PgHMGR1 sequence.
Supplemental Figure S2. PgHMGR2 sequence.
Supplemental Figure S3. DNA structures of PgHMGR1 and PgHMGR2.
Supplemental Figure S4. Motif analysis of HMGRs.
Supplemental Figure S5. Multiple alignments of HMGRs.
Supplemental Figure S6. Ginsenoside contents by inhibitor and activator.
Supplemental Figure S7. Ginsenoside contents by dark treatment.
Supplemental Figure S8. Structural features of PgHMGR1.
Supplemental Table S1. Primer sequences used.

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