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RESEARCH ADVANCE OF 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A SYNTHASE IN PLANT ISOPRENOID BIOSYNTHESIS

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ABSTRACT

Terpenoids are synthesized in plants via the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways, with isopentenyl diphosphate (IPP) as the main intermediate metabolite. 3-Hydroxy-3-methylglutaryl-Coenzyme A synthase(HMGS) is the second enzyme in MVA pathway of isoprenoid biosynthesis, and catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to yield HMG-CoA.A growing body of evidence now indicates that HMGS plays significant roles in biosynthesis of terpenoids in plants. The sequences and structures of *HMGS* genes isolated from most plants are highly homologous. *HMGS* has been found to be expressed in most organs of plants, and its expression correlates strongly with accumulation of terpenoids in plants. This review focus on the research progress in the biological significance, protein structure, regulatory mechanism, gene characterization and functional analysis of HMGS in plant. The studies on the gene family encoding HMGS has provided valuable insights into its function, phylogeny, and regulation of terpenoid content in plants.

Key words: Terpenoids; HMGS; HMG-CoA; biosynthetic pathway; expression.

INTRODUCTION

Terpenoids are the most diverse class of secondary metabolites in plants (Yonekura-Sakakibara and Saito, 2009). More than 25000 types of terpenoids have been identified so far in plants. Terpenoids possess multiple ecophysiological functions, such as attracting pollinators, regulating plant growth and development, regulating heat tolerance of plants, resisting photooxidative stress, and providing direct and indirect plant defense (Tholl, 2006). In addition, terpenoids have been extensively utilized in spices, cosmetics, food, drug and pesticides as important raw materials,. Therefore, terpenoids possess great commercial value (Chadwick *et al*., 2013).

Terpenoids are synthesized in plants by two pathways, MVA (mevalonate) and MEP (2C-methyl-D-erythritol 4-phosphate). As shown in Fig. 1 (Rodriguez-Concepcion and Boronat, 2002), the MVA pathway exists in the cytosol, while the MEP pathway exists in plastids (Lichtenthaler *et al*., 1997). Acetyl-CoA serves as substrate in the MVA pathway to synthesize the critical precursor MVA, which is subsequently converted into dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). IPP and DMAPP are the synthetic precursors of secondary metabolites such as terpenoids including steroids (Buhaescu and Izzedine, 2007). Since this pathway is also related to the synthesis of substances such as cholesterol that affect human health, it has been studied for a long time (Miziorko, 2011). The MEP pathway was found in plants more than twenty years ago (Lichtenthaler, 1999). In this pathway, pyruvate and glyceraldehyde-3-phosphate serve as substrates, which are catalyzed by seven enzymes to synthesize IPP and DMAPP.

Plants produce many types of terpenoids that are all generated from the same precursors: IPP and its isomer DMAPP, which are catalyzed by their corresponding enzymes to synthesize geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyldiphosphate (GGPP; Tholl, 2006). The intermediates GPP, FPP and GGPP are catalyzed by the corresponding terpene synthase to produce all isoprenoid end products (Patra *et al*., 2013; Vranová *et al*., 2013). The two pathways of terpenoid synthesis in plants are not fully independent, as evidence of cross-talk between IPP in the cytosolic and plastidial pathways have been found (Hemmerlin *et al*., 2003; Laule *et al*., 2003; Dudareva *et al*., 2005). As the first catalyzing enzyme in the MVA pathway, HMGS plays an important role in the biosynthesis of terpenoids in plants (Rodriguez-Concepcion and Boronat, 2002). Recently, Liao *et al*. (2014) summarized the past investigations on eukaryotic HMGS with particular focus on advance of plant HMGS study by researchers in China. To provide more detailed progress in plant HMGS research, this article provides a

comprehensive review on research conducted to date on the biological significance, catalytic mechanism, its regulatory mechanism and cloning, and functional analysis of HMGS in plants.

Biological significance of plant HMGS in the MVA pathway: As an important condensing enzyme in the MVA pathway, HMGS can catalyze condensation of acetyl-CoA and acetoacetyl-CoA to generate HMG-CoA, which is further converted into generate MVA by HMGR. The IPP of C5 skeleton is generated by pyrophosphorylation and decarboxylation of MVA, and the common precursors are supplied for the synthesis of tepenoid compounds such as mono-, sesqui-, di- and triterpenoid (McGarvey and Croteau, 1995). Rudney and Ferguson (1959) first showed that HMGS participates in the synthesis of polyisoprene and other researchers later confirmed that HMGS is involved in the second step of the catalysis through the MVA pathway (Chun *et al*., 2000a, 2000b).

HMGS can be classified broadly into cytosolic and mitochondrial forms (Clinkenbeard *et al*., 1975a, 1975b). The mitochondrial form of HMGS is only found in mammals and responsible for the synthesis of ketone bodies (Casals *et al*., 1992; Thompson *et al*., 1997). Its product HMG-CoA is broken down into acetoacetate and 2-hydroxybutyrate by HMG-CoA lyase. As the second enzyme in the MVA pathway, the cytosolic form of HMGS can catalyze acetyl-CoAto generate HMG-CoA. In addition, MVA pathway synthesizes mevalonic acid and isoprenoids (Miziorko, 2011). Since the MVA pathway exists in nearly all eukaryotes, further research is being done on the cytosolic form of HMGS than on the mitochondrial form. The entry of acetyl-CoA into the MVA pathway is controlled by HMGS through generating HMG-CoA which is utilized by HMGR.

Structure of plant HMGS protein: The plant HMGS protein is generally composed of 460-500 amino acid residues and has a relative molecular mass of 50-60 kDa (Argout *et al*., 2008; Schnable *et al*., 2009; Schilmiller *et al*., 2009; Zhang *et al*., 2011; Kai *et al*., 2013). The polypeptide chain of HMGS in plants contains three domains, N-terminus, catalytic region and C-terminus. The N-terminus of HMGS in mitochondrial from contains a conservative signal peptide sequence, which mediates transport of HMGS from the cytoplasm, where it is synthesized, to the mitochondria, and shows a high degree of similarity across most plants (Hegardt, 1999). The C-terminus of HMGS contains an important catalytic cysteine residue that acts as a nuclephile in the first step of reaction: the acetylation of the enzyme by acetyl-CoA (its first substrate) to produce an acetyl-enzyme thioester, releasing the reduced coenzyme A. The subsequent nucleophilic attack on acetoacetyl-CoA (its second substrate) leads to the formation of HMG-CoA (Theisen *et al*., 2004). Previous results have revealed that plant HMGS proteins are highly similar in their 3-D structures, which consist of two structural regions referred to as the upper and lower regions, similar to the HMGS from *Staphylococcus aureus* (Figure 2; Campobasso *et al*., 2004). The upper region is built around a five-layered core structure, - - - - , in which each comprises two helices and each is a mixed -sheet. The lower region does not contain any substructure or pseudo-symmetry. However, the interface of the upper and lower regions defines the acetoacetyl-CoA-binding sites (Theisen *et al*., 2004).

Most HMGS proteins contain a conserved motif 'NxD/NE/VEGI/VDx(2)NACF/YxG', which is considered to be important for HMGS function (Figure 3). This motif is localized at the entrance of the active site and plays an important role in controlling the catalysis of substrates by HMGS. Mutation of this motif has been found to decrease the catalytic activity of the enzyme or to lead to formation of abnormal products. In addition, three amino acid residues, namely Cys129, His264 and Asn326, in HMGS are essential for its catalytic activity (Misra *et al*., 2003). The earlier report of Misra *et al*. (1993) showed that Cys129 participates in the formation of the intermediate compound acyl-S-enzyme as the first step of catalytic reaction of HMGS. Misra and Miziorko (1996) further showed that His264 binds directly to acetoacetyl-CoA in the second step. Based on the results of mutation analysis, Sirinupong *et al*. (2005) indicated that Asn326 also has an important effect on HMGS activity. In addition, Pojer *et al*. (2006) determined the structure of BjHMGS1 by using protein X-ray crystallography. Cys117, His247 and Glu83 in BjHMGS1form a catalytic group common in HMGS to finish the three-stepreaction to produce HMG-CoA. These findings have deepened our understanding of the correlation between the structure and function of HMGS, which also provide important basic data for further studying the metabolic process of terpenoids.

Regulatory mechanism of HMGS in plants: HMGS participates in the synthesis of precursors of isoprenoids and provides reactive substrates for HMGR. HMGS and HMGR in plants and animals are synergistically regulated by feedback at multiple levels. For example, Schidler *et al*. (1985) firstly found that the specific inhibition of MVA-derived isoprenoid biosynthesis with mevinolin in radish might attributed to activity reduction of HMGS and HMGR. Subsequently, Dooley *et al*. (1998) demonstrated that HMGS as a key regulatory enzyme in the pathway for endogenous cholesterol synthase, was a target for negative feedback regulation by cholesterol. Moreover, Nagegowda *et al*. (2004) reported that *Brassica juncea* HMGS was inhibited by both products (HMG-CoA and CoASH) and one of the substrates (AcAc-CoA). Recently, Vranová *et al*. (2013) have also concluded that HMGS down-regulated at

translational level possibly by MVA, which triggers a negative-feedback loop. The results mentioned above implied that HMGS in higher plants might be is also under synergistic feedback regulation by several secondary metabolites such as isoprenoids. There are two types of feedback regulation of HMGS by metabolites (Dooley *et al*., 1998). The first is regulation by isoprenoids such as phytosterols on the cytosolic form of HMGS, and the second is regulation by fatty acid on the mitochondrial form of HMGS. Besides the multi-level feedback regulation by metabolites, HMGS is also under cross-regulation through other physiological pathways. For example, HMGS plays a role in the defense reaction of plants. Alex *et al*. (2000) reported that *HMGS* expression could be induced by wounding, methyl jasmonate (MJ) and salicylic acid (SA), and downregulated under abscisic acid (ABA)-induced stress and drought in *B. juncea*. More recently, several groups (Zhang *et al*., 2011; Kai *et al*., 2013) also demonstrated that SA and MJ could up-regulate *HMGS*expression in*Camptotheca acuminate*,and *Salvia miltiorrhiza*,respectively. In addition, the activity and expression of HMGS are also regulated by circadian rhythm. For example, Suwanmanee *et al*. (2004) found that the activity and mRNA expression level of HMGS in rubber latex exhibit diurnal variation, with the highest peak occurring at 02:00 AM. It is well known that blossoming of many plants and release of terpenoids by flowers follow circadian rhythm. This specific change in the release of terpenoids may be related to the time of appearance of pollinators. Therefore, we speculate that the following of circadian rhythm by *HMGS* expression may be related to the release of terpenoids in plants. In addition to these exogenous induction factors, *HMGS* expression also varies with developmental stage and differentiation. Alex *et al*. (2000) studied the developmental expression pattern of *HMGS* in the flower, seed and seedling of *B. juncea*, and found that *HMGS* expression was the highest at early stages in these parts. In addition, *HMGS* expression showed correlation with rapid cell division and growth. For example, Nagegowda *et al*. (2005) observed that predominant localization of HMGS mRNA in the stigmas and ovules of flower buds and in the piths of seedling hypocotyls of *B. juncea*. The expression pattern of *HMGS* in plant tissues varies greatly across different plants. For example, *HMGS* is mainly expressed in needles, stems, hypocotyls and cotyledons, but seldom expressed in roots of *C. acuminata* (Kai *et al*., 2013) and *Taxus* × *media* (Kai *et al*., 2006) whereas *HMGS* is constitutively expressed in the leaf, stem and root of *S. miltiorrhiza* (Zhang *et al*., 2011). Thus, the discovery of organ specificity and developmental regulation of *HMGS* expression provides a basis for searching the specific promoter that regulates *HMGS* expression and terpenoid accumulation.

Molecular cloning and functional analysis of the *HMGS* gene in plants: Due to its importance for terpenoid biosynthesis, HMGS is one of the most extensively studied enzymes in the MVA pathway. Since the first plant *HMGS* gene was cloned from *Arabidopsis thaliana* in 1995 (Montamat *et al*., 1995), scientists have cloned *HMGS* genes from almost 40 plants, including crops, conifers, medicinal plants, spice plants, ornamental plants and model plants (Table 1). In order to better understand the phylogenetic relationship among HMGS proteins in different plants, cluster analysis of phylogenetic tree was conducted based on the HMGS protein sequences of 29 plants. As shown in Figure 4, the HMGS sequences of phycophyta and higher plants belong to two separate branches. *Chara vulgaris* is a type of lower plant, and its *HMGS* gene is independent of other gene families. Higher plants in the phylogenetic tree are clustered into gymnosperms and angiosperms, and angiosperms are further clustered into monocotyledons and dicotyledons. This indicates that HMGS is conserved in terms of evolutionary origin across different plants, and shows conservation of amino acid sequence and functional domain.

Montamat *et al*. (1995) cloned the first *HMGS* gene from *A. thaliana*, and conducted yeast complementation tests to confirm that AtHMGS has catalytic activity. Later, Wegener *et al*. (1997) cloned an *HMGS* gene induced by ozone in *Pinus sylvestris*, and showed that ozone might regulate isoprenoid biosynthesis by upregulating *PsHMGS* expression. Alex *et al*. (2000) cloned an *HMGS* gene regulated by organ development in *B. juncea* and induced by exogenous abiotic treatment. This indicates that *HMGS* may participate in the resistance of plants to environmental stresses. Results of further transgenic experiments indicated that overexpression of *BjHMGS* in *A. thaliana* not only increased sterol concentration, but also enhanced *Botrytis cinerea* resistance and H_2O_2toleranceand promoted germination in the transgenic plants (Wang *et al*., 2012). Likewise, Ishiguro *et al*. (2010) studied the molecular mechanism of HMGS involved in development of tapetum-specific organelles and fertility of pollen grains, and found that the MVA pathway is essential for development of both tapetosomes and elaioplasts in tapetal cells and for pollen viability, at least during pollen tube elongation. Taken together, these findings indicate that HMGS plays an important role in the development and defense mechanism of plants.

The rubber secreted by *Hevea brasiliensis* is an important industrial material and also a type of terpenoids. Suwanmanee *et al*. (2002) first cloned the *HMGS* gene in *H. brasiliensis* and further research indicated that the levels of mRNA and activity of *HMGS* in *H. brasiliensis* are closely related to accumulation of rubber in the plants. Subsequently, Sirinupong *et al*. (2005) also isolated another *HMGS* gene, *HMGS2*, in *H.*

brasiliensis, and the homology of nucleotide and amino acid sequences between *HMGS1* and *HMGS2* was found to be 92% and 94%, respectively. RT-PCR analysis showed that expression of *HMGS2* in latex-producing cells and petioles were significantly higher than that in leaves, which suggests that *HMGS1* and *HMGS2* are two critical genes in the synthetic pathway of latex. Besides *H. brasiliensis*, most of the current studies on the function of *HMGS* in plants have focused on its effect on the metabolism of medicinal ingredients. For instance, Zhang *et al*. (2011) and Kai *et al*. (2006, 2013) isolated the *HMGS* genes from three medicinal plants *T. media*, *C. acuminata*, and *S. miltiorrhiza*. RT-PCR analysis revealed that *HMGS* genes are expressed in tissue-specific manner and can be induced by exogenous factors, including SA and MJ, in these plants. However, the roles and regulatory mechanisms of *HMGS* genes in the biosynthesis of active constituents, including taxol, camptothecin and tanshinones, need to be further investigated in medicinal plants.

Table 1. The protein information of HMGS cloned in plants to date.

Protein name	GenBank accssion No.	Plant species	References
BjHMGS	AAF69804	*Brassica juncea*	Alex *et al*., 2000
SmHMGS	ACV65039	*Salvia miltiorrhiza*	Zhang *et al*., 2011
CaHMGS	ACD87446	*Camptotheca acuminata*	Kai *et al*., 2013
HbHMGS	AAK73854	*Hevea brasiliensis*	Suwanmanee *et al*., 2004
TmHMGS	AAT73206	*Taxus×media*	Kai *et al*., 2006
AtHMGS	CAA58763	*Arabidopsis thaliana*	Montamat *et al*., 1995
CrHMGS	XP_006287702	*Capsella rubella*	Slotte *et al*., 2013
PsHMGS	CAA65250	*Pinus sylvestris* L	Wegener *et al*., 1997
TcHMGS	EOY24602	*Theobroma cacao*	Argout *et al*., 2008
PtHMGS	EEE79437	*Populus trichocarpa*	Tuskan *et al*., 2006
VvHMGS	CBI34763	*Vitis vinifera*	Jaillon *et al*., 2007
SgHMGS	AEM42970	*Siraitia grosvenorii*	Tang *et al*., 2011
ZmHMGS	DAA40580	*Zea mays*	Schnable *et al*., 2009
AaHMGS	ACY74339	*Artemisia annua*	Graham *et al*., 2010
CvHMGS	ABO27206	*Chara vulgaris*	Grauvogel and Petersen, 2007
SlHMGS	XP_004252572	*Solanum lycopersicum*	Schilmiller *et al*., 2009
RcHMGS	EEF51079	*Ricinus communis*	
CsHMGS	AFC34137	*Camellia sinensis*	
GmHMGS	XP_003549866	*Glycine max*	
PpHMGS	EMJ11192	*Prunus persica*	
PgHMGS	ADI80347	*Panax ginseng*	
CrHMGS	AEC13715	*Catharanthus roseus*	
FvHMGS	XP_004298742	*Fragaria vesca subsp. vesca*	
NNHMGS	ABV02025	*Nicotiana langsdorffii* x *Nicotiana sanderae*	
CmHMGS	XP_004511614	*Cicer arietinum*	
MtHMGS	XP_003611167	*Medicago truncatula*	
PkHMGS	AFP23864	*Picrorhiza kurrooa*	
SiHMGS	XP_004957395	*Setaria italica*	
BdHMGS	XP_003574875	*Brachypodium distachyon*	
OsHMGS	EAZ09792	*Oryza sativa* Indica Group	

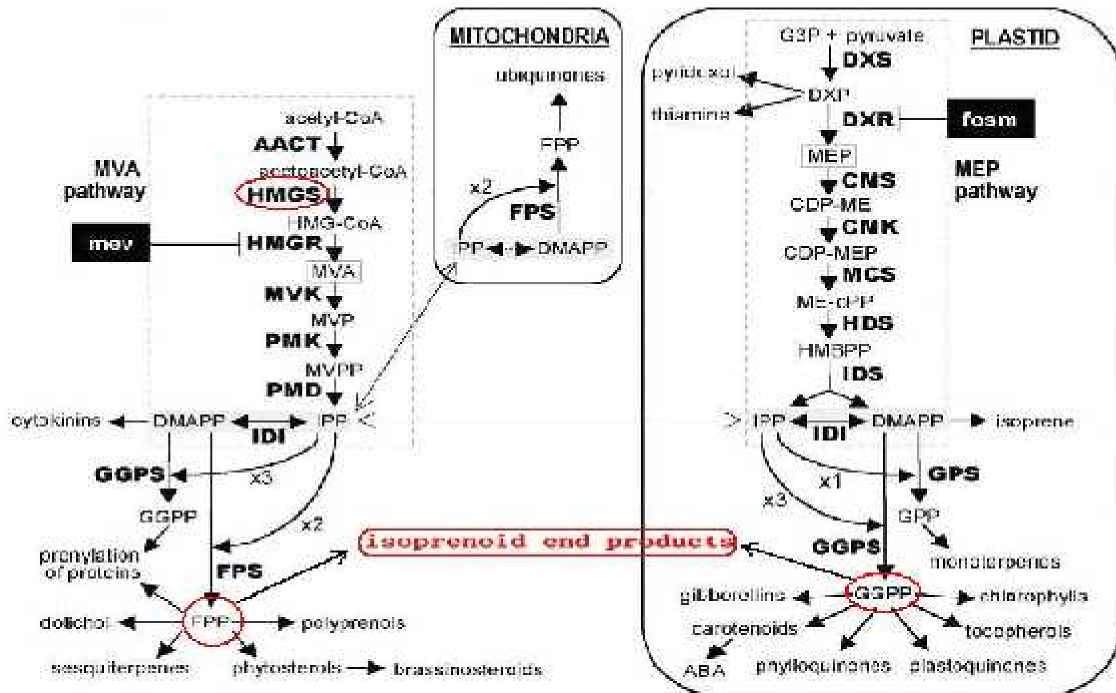

Figure 1. Isoprenoid biosynthesis pathway in the plant cell (Rodriguez-Concepcion and Boronat, 2002). Enzymes are indicated in bold: AACT, acetoacetyl CoA thiolase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MVK, MVA kinase; PMK, MVP kinase; PMD, MVPP decarboxylase; IDI, IPP isomerase; GPS, GPP synthase; FPS, FPP synthase; GGPS, GGPP synthase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; CMS, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-(Cytidine 5 -diphospho)-2-C-methyl-D-erythritol kinase; MCS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; IDS, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase.

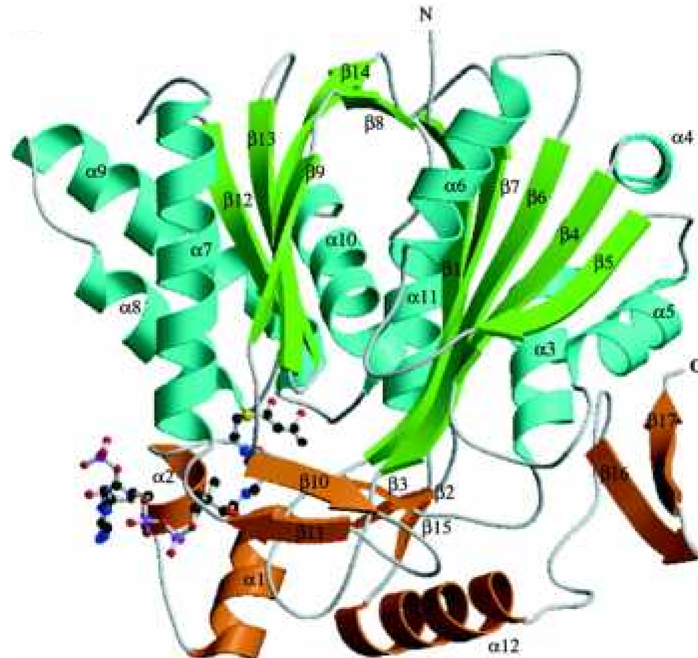

Figure 2. The 3D structure of HMGS with strands and helices (Campobasso *et al*., 2004). -sheets are green; -helices are cyan; the lower region is colored orange to distinguish it from the upper region.

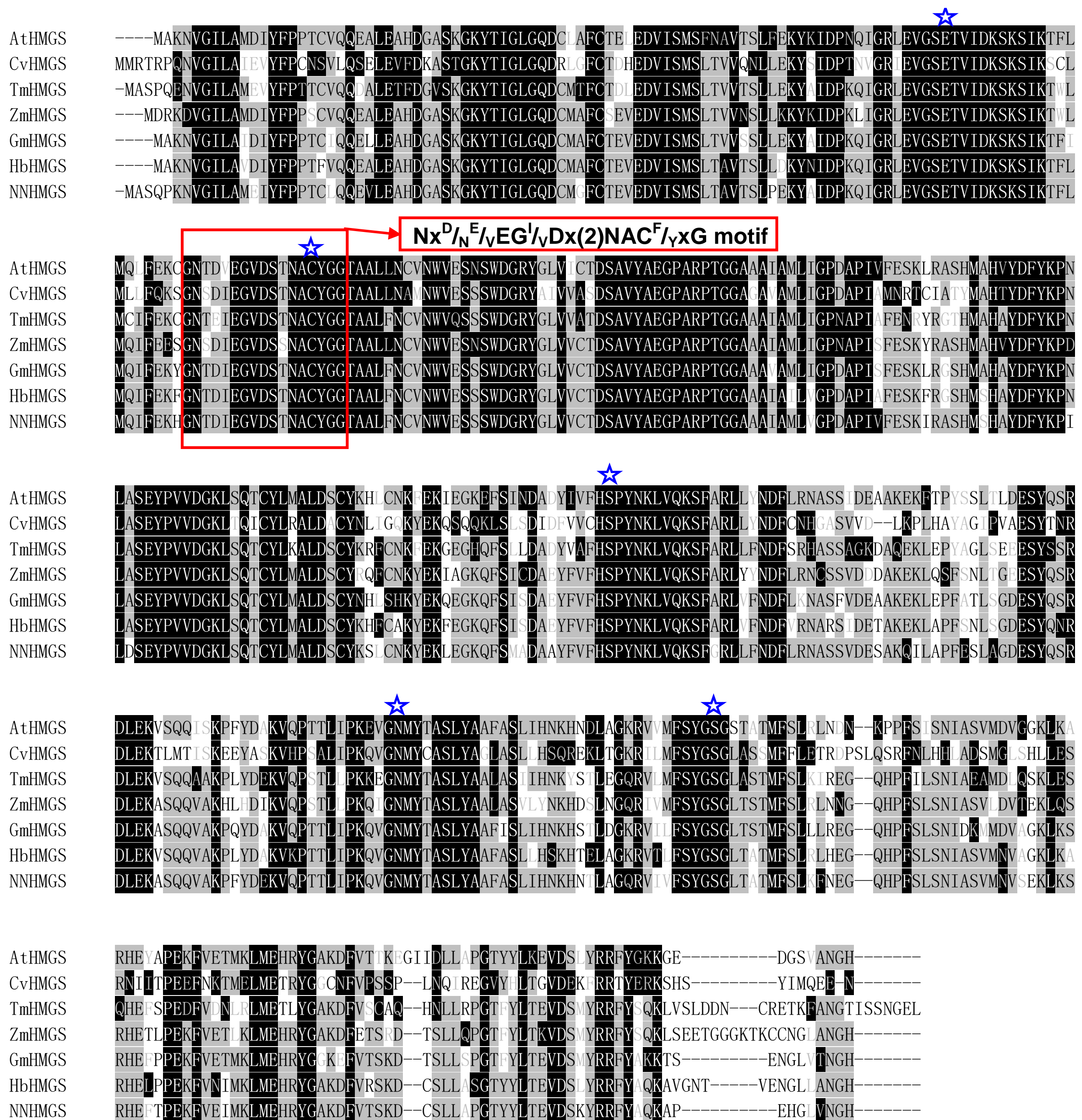

Figure 3. Multialignment of the amino acid sequences of HMGS from different plants. The identical amino acids are indicated with white foreground and black background. The conserved amino acids are indicated with black foreground and gray background. Non-similar amino acids are indicated with black foreground and white background. The conservative motif, 'NxD/NE/VEGI/VDx(2)NACF/YxG' responsive for catalytic activity is boxed. The active sites is indicated with star. The GenBank accession numbers of these sequences are showed in Table 1.

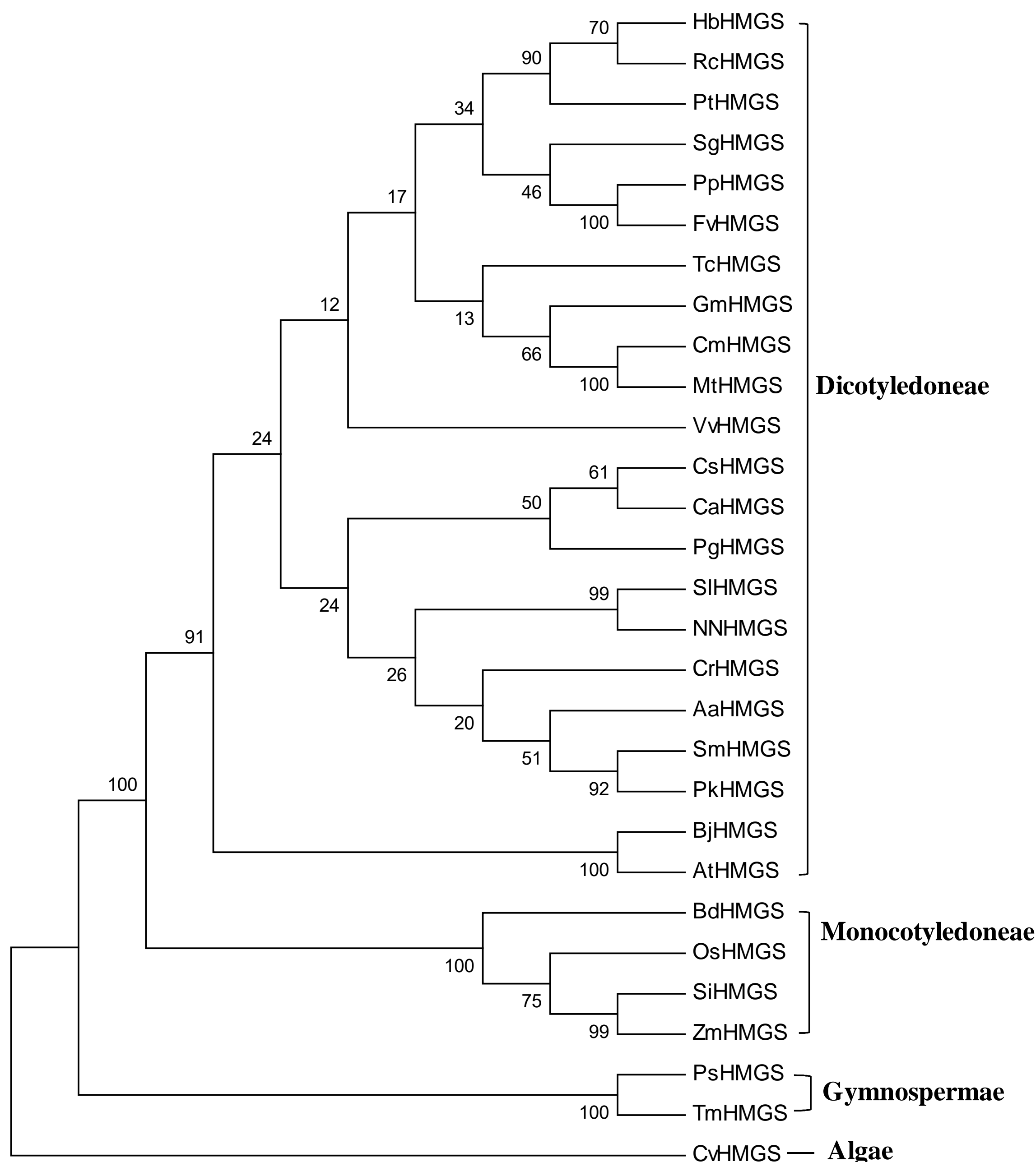

Figure 4. The phylogenetic analysis of HMGSs from different plant species by MEGA5 from Clustal W alignments. The number for each interior branch is the percent bootstraps value (100 replicates). The GenBank accession numbers of these sequences are showed in Table 1.

Conclusion: Terpenoids not only play an important role in plant physiology, but also have wide applications in fields such as industry, medicine and health. Therefore, regulation of biosynthesis of terpenoids in plants is an active area of research. Significant progress has been made in our understanding of regulation and function of *HMGS* and its homologous genes in the past decade. However, regulation of the metabolic network involved in terpenoid metabolism in plants, especially the transcriptional regulation of enzymes (including HMGS) involved in terpenoid metabolism, requires further investigation. More specifically, the mechanism by which HMGS regulates synthesis of terpenoids at transcriptional and translational levels remains to be explored further. In addition, promoting research on the interaction network of critical proteins in the terpenoid synthetic pathway in plants is highly important.

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