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Characterization of 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (HDS) gene from *Ginkgo biloba*

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Abstract Diterpene trilactone ginkgolides, one of the major constituents of *Ginkgo biloba* extract, have shown interesting bioactivities including platelet-activating factor antagonistic activity. 1-Hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (HDS), converting 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate into 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate, is the penultimate enzyme of the seven-step 2-*C*-methyl-D-erythritol 4-phosphate pathway that supplies building blocks for plant isoprenoids of plastid origin such as ginkgolides and carotenoids. Here, we report on the isolation and characterization of the full-length cDNA encoding HDS (*GbHDS*, GenBank accession number: DQ251630) from *G. biloba.* Full-length cDNA of GbHDS, 2,763 bp long, contained an ORF of 2,226 bp encoding a protein composed of 741 amino acids. The theoretical molecular weight and p*I* of the deduced mature GbHDS of 679 amino acid residues are 75.6 kDa and 5.5, respectively. From 2 weeks after initiation of the culture onward, transcription level of this gene in the ginkgo embryo roots increased to about two times higher than that in the leaves. GbHDS was predicted to possess chloroplast transit peptide of 62 amino acid residues, suggesting its putative localization in the plastids. The transient gene expression in *Arabidopsis* protoplasts confirmed that the transit peptide was capable of delivering the GbHDS protein from the cytosol into the chloroplasts. The isolation and characterization of *GbHDS* gene enabled us to further understand the role of GbHDS in the terpenoid biosynthesis in *G. biloba*.

Keywords Ginkgolides · *Ginkgo biloba* · 1-Hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase · Isoprenoid · MEP pathway · Terpene

Introduction

Terpenoids in living organisms are an extremely diverse group of compounds taking roles ranging from pigments and fragrances to vitamins and precursors of the sex hormones [1]. Though found in all organisms, terpenoids are especially abundant and diverse in plants [2, 3]. Surprisingly, the structural diversity of terpenoids is derived from the simple five-carbon building units of the isoprene carbon skeleton, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) [4]. Terpenoids are thus also known as isoprenoids. In plants, two pathways for the synthesis of the isoprene building blocks are in operation: cytosolic mevalonate (MVA) pathway starting from 3 acetyl-CoA to finally yield IPP through six-step reactions and plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway simultaneously producing IPP and DMAPP from pyruvate and D-glyceraldehyde 3-phosphate (G-3-P)

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through seven serial reactions (Fig. 1) [5]. Though both pathways produce the same isoprenoid building blocks, the channeling of the isoprene units to the end products in plants is known to be specific: mono-, di-, and tetra-terpenoids originate from the MEP pathway, whereas sesqui- and triterpenoids are from the mevalonate pathway. However, evidences of cross-talks between the pathways have been reported [6, 7].

Ginkgolides, highly modified diterpene lactones found uniquely in *Ginkgo biloba*, have many pharmacological activities such as the anti-platelet-activating factor (PAF) activity [8] and the selective glycine receptor antagonism [9]. Though ginkgolides accumulate in the Ginkgo leaves, they are known to be synthesized in the roots and subsequently translocated to the leaves [10]. Studies have demonstrated that building blocks for the ginkgolides are derived through the MEP pathway [11]. Despite the impressive progress on the structural determination and biosynthetic pathway of ginkgolides, much work still remains on the enzymology and molecular biology involved in the ginkgolide biosynthesis. Nevertheless, some achievement has been reported. Geranylgeranyl diphosphate (GGPP) synthase gene was cloned from *G. biloba* [12], and levopimaradiene synthase (LPS) gene that encodes enzyme converting GGPP into levopimaradiene, the precursor of ginkgolide, was cloned and functionally characterized [13]. Our group isolated all MEP pathway genes from *G. biloba* except for *HDS*, demonstrated their functional activities, and determined the temporal and spatial transcription patterns of each gene [14–19]. These works showed that there are two additional multi-copy genes in the MEP pathway of *Ginkgo* besides *DXS*, which is the well-known multi-copy gene in higher plants [18, 20]. *GbCMEK* and *GbIDS* each have two and three copies of the isogene, respectively [16, 19]. The *IDS* gene is also present in multi-copy in other gymnosperm plants such as

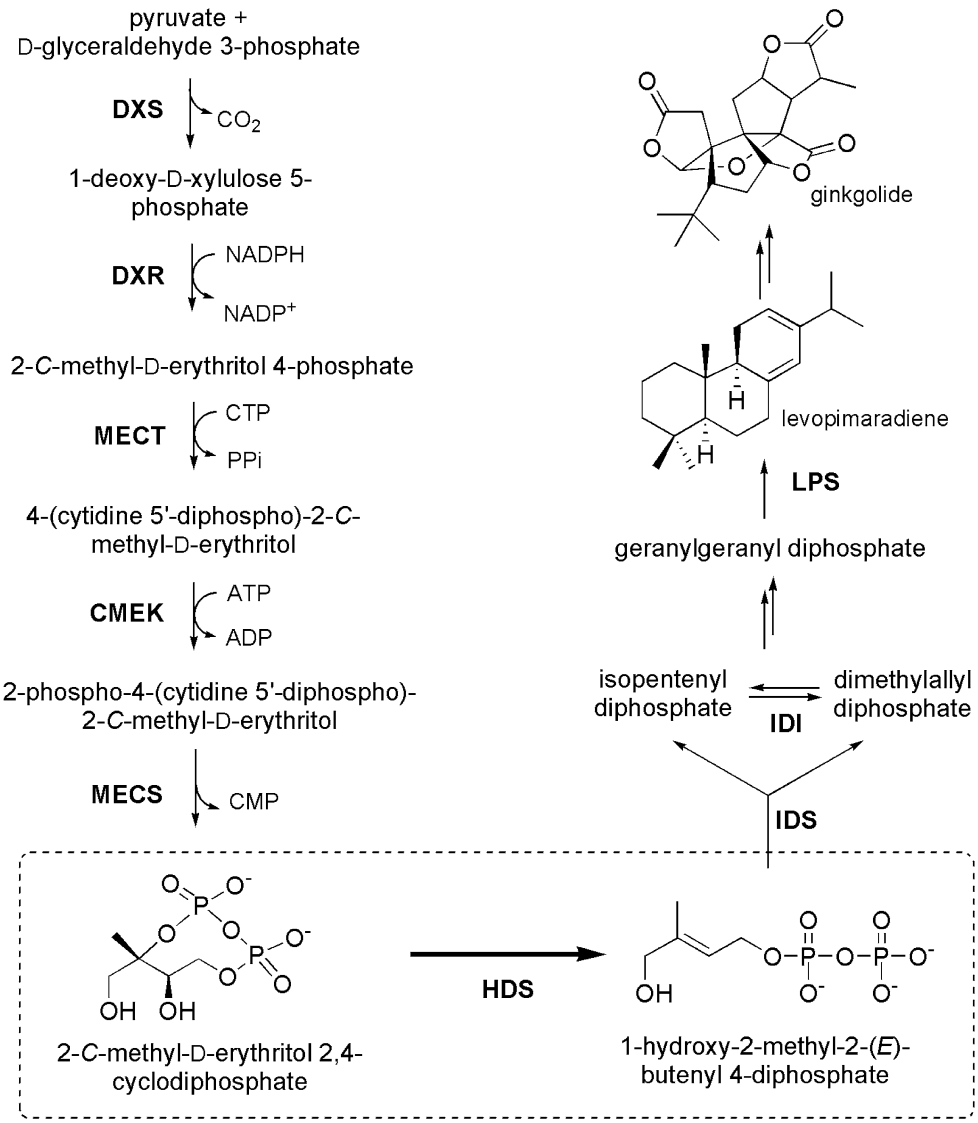

Fig. 1 2-*C*-Methyl-D-erythritol 4-phosphate (*MEP*) pathway in the isoprenoid biosynthesis of *G. biloba*. DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MECT, 2-*C*-methyl-D-erythritol 4-phosphate cytidyltransferase; CMEK, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol 4-phosphate kinase; MECS, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; IDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase; IDI, isopentenyl diphosphate isomerase; LPS, levopimaradiene synthase

Pinus [16, 20, 21] and *Cycas* [16], whereas two-copy *GbCMEK* is unique in the plant EST database [19].

1-Hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (HDS), the penultimate enzyme in the MEP pathway sequence, converts 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) into 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate (HMBPP). Up until now, except for a few examples including tomato [22], *Arabidopsis* [23], and *Hevea brasiliensis* [24], HDSs from plants have not been fully characterized. The *HDS* gene in tomato showed no significant change in the transcript number at different stages of the fruit development, although a huge supply of the isoprenoid precursors through the MEP pathway is required during the development. Furthermore, little difference was found in the transcript levels of *HDS* in both the wild-type and carotenoid-depleted mutant fruits, an indication that in tomato fruit this gene is not regulated by the end product carotenoids [22]. *Arabidopsis clb4* mutant, a defective *HDS* mutant, showed the arrested chloroplast development at the proplastid stage, indicating this gene is required for the early chloroplast development in *Arabidopsis*. The non-cell autonomous nature of the *clb4* mutant suggests a movement of the isoprene building blocks from cytoplasm to the chloroplasts [23]. Recent work demonstrated that CSB3 *Arabidopsis* mutant, a recessive partial loss-of-function mutant of the *HDS* gene, showed a high level of resistance to the biotrophic pathogens *Pseudomonas syringae* and *Plectosphaerella cucumerina* due to the activation of the plant defenses [25].

In the present work, we report the cloning and characterization of the *HDS* gene (*GbHDS*, GenBank accession number: DQ251630) from *G. biloba*. Intracellular localization of GbHDS and the transcription pattern of the gene with respect to the ginkgolide biosynthesis were also evaluated.

Materials and methods

Plant materials

Dehulled seeds of ginkgo, purchased from Sillim Market, Seoul, Korea, were sterilized in 4% NaOCl solution for 20 min. After extensive washing in distilled water, the embryos were separated from the cotyledons. The embryos were then placed on the hormone-free MS medium and incubated in a controlled-growth chamber (23°C under 16 h/8 h light and dark regimen). Roots and leaves from the seedling were harvested every week for the transcription analysis. For the *Arabidopsis* protoplast isolation, the plants were grown in the same controlled-growth chamber for 4 weeks.

RNA isolation and cDNA synthesis

Total RNA and mRNA were isolated from the roots of 1-month-old embryo culture through the CTAB method [26]. For the full-length single strand cDNA synthesis, GeneRacer Kit (Invitrogen, http://www.invitrogen.com) was used with 1 μg of the isolated mRNA following the manufacturer's protocol. In reverse transcription PCR (Qiagen Omniscript Reverse Transcription kit), 2 μg of total RNA from each plant sample was used to synthesize the single strand cDNAs, which were then used in quantitative real-time polymerase chain reaction (QRT-PCR).

Isolation of full-length cDNA of GbHDS by RACE

All primers used in this research are listed in Table 1. A degenerate primer pair, GcpE-F and GcpE-B, was designed based on the conserved regions of the previously known plant *HDS* genes and used to amplify the core cDNA fragment. The PCR fragment was cloned into the pGEM-T easy vector (Promega) and sequenced. For the full-length cDNA isolation, rapid amplification of cDNA end (RACE) PCR method was used. The primer pair of GeneRacer 5′-nested and HDS-5B1 was used for 5′-RACE, whereas the primer pair of GeneRacer 3′-nested and HDS-3F1 was used for 3′-RACE. Each RACE product was cloned and sequenced as mentioned above. Based on each RACE sequences, a full-length cDNA sequence of *HDS* was assembled and amplified with its respective primer pair, HDS-TE-START and HDS-TE-STOP.

Multiple alignment and bioinformatic analyses

The deduced amino acid sequence of GbHDS was aligned with the sequences of other known plant HDSs from GenBank using the Multalin program (http://prodes.toulouse.inra.fr/multalin/). Prediction of the chloroplast-targeting peptides was made using ChloroP program (http://

Table 1 List of primers used in this study

Primer	Sequence (5′ → 3′)
GcpE-F	GCAATGCGTATTGGAACAAA
GcpE-B	CATTCACAATGCANCCCATGAT
HDS-3F1	CGGAGTATGTCTCATGCCCATCT
HDS-5B1	TGCCTGAACCATGACTACAGGATT
HDS-F	ATTGATCCTTGTAGGAGACT
HDS-B	TCTGTAATCCACTTCTTCAC
HDS-TE-START	GGATCCA ATGGCCGCTGGAACAA
HDS-TE-STOP	GGATCC CATTCTTCAACTGGTGGGT
HDS-STOP2	GGATCC CTGCCAAGTGCAACGTT

www.cbs.dtu.dk/services/ChloroP/). TreeTop program (http://www.genebee.msu.su/services/phtree_reduced.html) was used for the phylogenetic tree construction at a default setting using the cluster algorithm. The reliability of the tree was measured by bootstrap value with 100 replicates. Partial *HDS* sequences of *Picea abies* and *Pinus taeda* were collected from the DFCI Gene index database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi). Putative molecular weight and p*I* values were calculated using the Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). The SIM program (http://www.expasy.org/tools/sim-prot.html) was used for the comparison of each HDS protein sequence from GenBank.

Transcript profile analysis by QRT-PCR

Total RNA was separately extracted from the roots and leaves of *Ginkgo* embryo culture harvested every week for 4 weeks. The sampling was done in triplicate with three plants per sample. Single strand cDNA was synthesized from 2 μg of each isolated total RNA with oligo$(dT)_{17}$ primer as described previously [18]. The tissue-specific expression of *GbHDS* gene was examined with the gene-specific primer pair (HDS-F and HDS-B) by QRT-PCR. The PCR reaction was carried out in triplicates for 45 cycles on the Rotor-Gene 2000 Real Time Amplification System (Corbett Research, http://www.corbettresearch.com) using the Qiagen Quantitect SYBR Green PCR system [18].

Protein targeting analysis

The fragments encoding the full-length protein and the N-terminal 100 amino acid residues, were separately amplified, respectively with each primer pair (for full-length protein, HDS-TE-START and -STOP; for N-terminal, HDS-TE-START and -STOP2) and fused to the psmGFP vector in frame. *Arabidopsis* protoplasts were prepared from the leaves of 4-week-old seedling. The constructed plasmids were transformed into the *Arabidopsis* protoplasts using a polyethylene glycol method [27]. In brief, 20 μg each plasmid DNA (1 μg/μl) was transfected into 300 μl protoplast suspension (10^6/ml), and the transfected protoplasts were incubated for 12–15 h at 22°C in darkness. The images were captured with the MRC-1024 Confocal Laser Scanning Microscope system (Bio-Rad). Fluorescence images of the protoplasts were taken on a confocal laser scanning microscope at 500–530 nm for GFP (green) and 600–660 nm for chlorophyll autofluorescence (red). Data were processed using the CAS program (Bio-Rad) and the Adobe Photoshop v7.0 software.

Results and discussion

Full-length cDNA isolation and sequence analyses of GbHDS

For the cloning of *GbHDS*, a pair of primer, designed based on the conserved region of *HDS* genes from different plants, was used for the amplification of the core sequence of *GbHDS*. A DNA fragment of 1,351 bp was amplified with this degenerate primer pair and was identified as a putative *HDS* by BlastX analysis. Based on this sequence information, two gene-specific primer pairs were designed and used in 5′- and 3′-RACE PCRs, respectively to obtain 1,070- and 681-bp fragments. Sequence analysis indicated that the full-length cDNA of *GbHDS* was 2,763 bp containing 164 bp 5′-untranslated region (UTR) and 193 bp 3′-UTR. This cDNA contained an open reading frame (ORF) of 2,226 bp encoding a protein consisting of 741 amino acids. ChloroP program predicted that GbHDS had the N-terminal chloroplast transit peptide consisting of 62 residues that is absent in the *E. coli* counterpart [28] and was similar in length to the transit peptides of the putative HDSs from other plants such as *Catharanthus roseus* (CrHDS), *Oryza sativa* (OsHDS), *Lycopersicon esculentum* (LeHDS), and *A. thaliana* (AtHDS) (on-line supplementary Fig. S1). The deduced amino acid sequence of the mature GbHDS consisted of 679 residues, and the theoretical molecular weight and p*I* were 75.6 kDa and 5.5, respectively. At the protein level, mature GbHDS without the predicted plastid signal peptide had about 85–86% sequence identities with the putative CrHDS, AtHDS, and OsHDS.

HDS is an iron–sulfur protein, which has a [4Fe–4S] cluster for catalysis [28]. The CXXC motif, the common binding motif for [4Fe–4S] cluster in all plant HDSs, was also present in GbHDS. Amino acid alignment showed the presence of a large additional domain of 268 residues (ca. 30 kDa) in the plant HDS protein [22] not present in the bacterial proteins (on-line supplementary Fig. S1). Querol et al. [29] reported that this additional domain, showing no significant homology with any other known proteins, did not affect the complementation assay with the *E. coli* HDR disruptant. Mature GbHDS, however, could not rescue the *E. coli* disruptant, NMW18 (pTMV20KM) [30] (data not shown). *H. brasiliensis* HDS (HbHDS) also could not complement the same *E. coli* HDS mutant [24]. Possibly the extra domain composed of 268 residues in plant HDS, putatively responsible in the interaction with the electron shuttle system, could be incompatible with the bacterial electron shuttle flavodoxin/flavodoxin reductase system in *E. coli* [31]. Another possibility is the difference in the codon usage between plant and *E. coli*. In particular, abundant arginine residues in the N-terminal regions of the

mature GbHDS were encoded by the AGA codon rare in *E. coli* [29].

Molecular evolution analysis

A phylogenetic tree was constructed using the deduced amino acid sequences of the plant HDSs retrieved from GenBank and DFCI Gene index database to trace the evolutionary relationship among the HDSs of different species (Fig. 2). The results clearly demonstrated that plant HDSs formed one large cluster separated from the bacterial HDSs. Within the plant group, GbHDS and *Pinus taeda* HDS, being of gymnosperm origin, formed a clade separated from an angiosperm clade. However, HDS from *P. abies* (PaHDS) was an outlier from gymnosperm clade, presumably because the partial sequence of PaHDS was used to construct the tree.

Profiles of *HDS* gene transcript levels among organs

The copy number of *GbHDS* transcripts in the 1-week-old embryo roots was slightly lower than that of the leaves (Fig. 3). However, the level in the roots was consistently higher than that in the leaves from the week 2 onward, and reached two times higher level at week 3. Interestingly, *Ginkgo* possesses multiple MEP pathway genes at three different steps at *DXS* [18], *CMEK* [19], and *IDS* [16] stages along the pathway. At least one copy from each of these genes, generically labeled as type 2 or class 2, was differentially transcribed in the roots in a much higher copy number than type 1 or class 1 isogene. On the other hand, the type 1 gene transcription was not specifically targeted—the type 1 gene appeared at comparable levels both in roots and leaves. This observation together with other evidences was interpreted as that the type 2 enzymes have correlation with the ginkgolide biosynthesis [16, 18, 19],

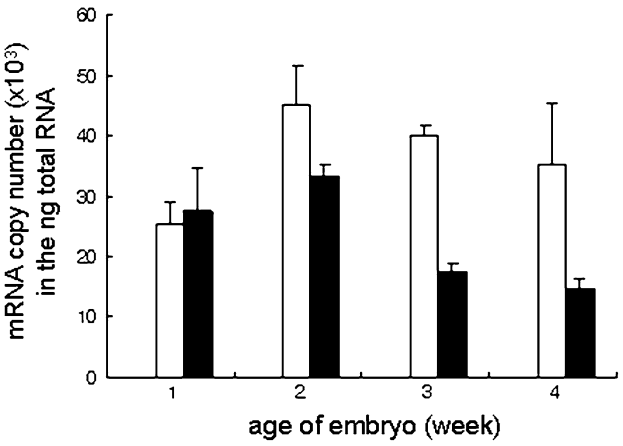

Fig. 3 Transcript levels of *GbHDS* in the *Ginkgo* embryos grown on the hormone-free MS medium for 4 weeks. *White bar*, root; *black bar*, leaf; *Y* axis, mRNA copy number ($\times 10^3$) per ng total RNA

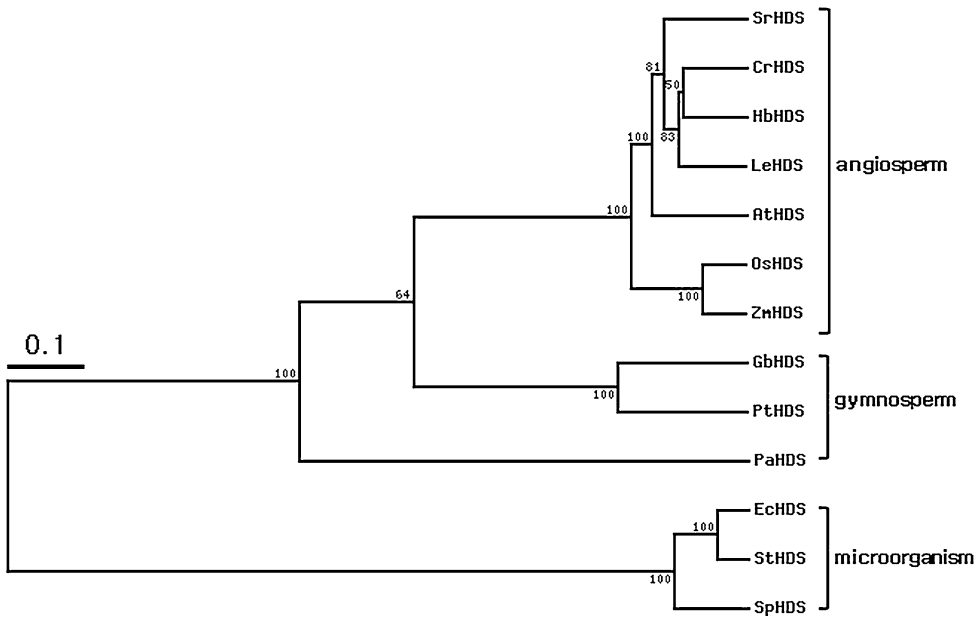

Fig. 2 Phylogenetic tree of HDSs. Bootstrap values are expressed in percentages and placed at the nodes in the tree. The *bar* on the *tree* represents the branch length equivalent to 0.1 amino acid changes per residue. Plant origin: AtHDS (*Arabidopsis thaliana*, accession no. AF434673), LeHDS (*Lycopersicon esculentum*, AAO15447), OsHDS (*Oryza sativa*, BAD19354), GbHDS (*Ginkgo biloba*, DQ251630), ZmHDS (*Zea mays*, AAT70081), CrHDS (*Catharanthus roseus*, AAO24774), SrHDS (*Stevia rebaudiana*, ABG75916), HbHDS (*Hevea brasiliensis*, AB294707), PaHDS (*Picea abies*, TC48506), PtHDS (*Pinus taeda*, TC91039). Bacterial origin: EcHDS (*E. coli*, AAC75568), StHDS (*Salmonella typhimurium* LT2, NP_461458), SpHDS (*Serratia proteamaculans* 568, YP_001479833)

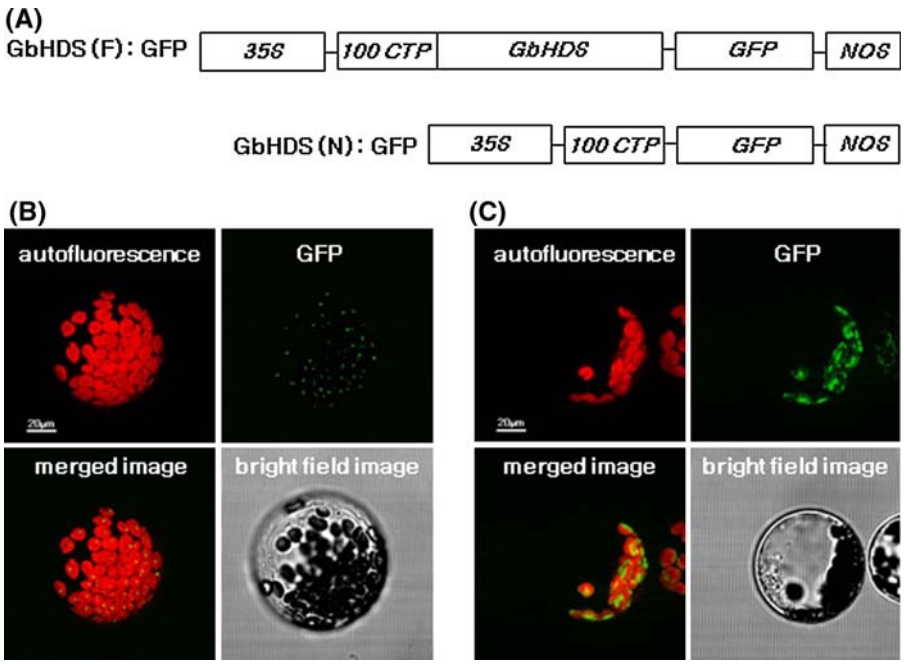

Fig. 4 Subcellular localization of the fusion constructs of N-terminal residues and full-length protein of GbHDS with GFP. **a** plasmid construction; **b** localization of GFP fused with full-length GbHDS; **c** localization of GFP fused with N-terminal 100 residues of GbHDS. 35S, 35S promoter; *CTP*, chloroplast transit peptide; *NOS*, *NOS* terminator

whereas the class 1 enzymes are performing the household function. The transcription pattern of the single-copy genes such as *GbDXR* [18] and *GbMECS* [15], except for *GbMECT* [17], appeared as hybrid between type 1 and 2 isogenes; they are transcribed at noticeably higher expression levels, but not in such a distinctive way as the type 2 enzymes, in the roots, suggesting a dual role in the primary and the secondary metabolisms. In fact, the pattern of transcription level of *GbHDS* during the culture period followed those of *GbDXR* and *GbMECS*, again indicating the dual role [15, 17, 18].

Intracellular targeting analysis of HDSs

Chloroplast transit peptide signals the delivery of premature protein synthesized in the cytosol into the chloroplast. At the target location, the transit peptide is removed to generate a fully functional mature protein [32]. *Ginkgo* HDS was predicted by the ChloroP program to have a chloroplast transit peptide consisting of 62 amino acid residues. All other MEP pathway proteins in the plant are known to have transit peptide. Length of the chloroplast transit peptide of the Ginkgo HDS was similar to those predicted for the other plant HDSs, even though its sequence similarities were very low in this region. To determine whether this putative transit peptide was functional, we constructed two plasmids that expressed GFP-fused proteins with the N-terminal 100 amino acids and a full-length sequence of GbHDS (Fig. 4a). Transient expression of the fused proteins in the *Arabidopsis* protoplasts showed that both the full-length HDS- and N-terminal 100 residue-fused GFPs appeared in the chloroplasts (Fig. 4b, c), indicating that HDS was imported into the chloroplast, in agreement with the proposed primary role of HDS in the MEP pathway for the biosynthesis of plastidic isoprenoids. The present finding suggests the possibility that the product of HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate (HMBPP) could occur in the cytosol, since one isozyme of the terminal MEP pathway enzyme IDS was suggested to occur in cytosol [16]. Because HDS was found to be localized only in the chloroplast, the putative occurrence of HMBPP in the cytosol would be a result of transport from chloroplast to cytosol. However, the transportation of HMBPP across the chloroplast membrane at a discernable level has not been demonstrated [6, 7]. Furthermore, the presence of GbCMEK1 in the cytosol was also indicated [19]. These findings alluded the possible occurrences of the whole later phase of MEP pathway in cytosol, which, however, is negated since GbMECS [15] and GbHDS were not found in the cytosol. Therefore, the presence of the MEP pathway intermediates and the enzymes in the cytosol need be vigorously verified through a more sensitive method.

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