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Cloning and functional characterization of 2-*C*-methyl-D-erythritol 4-phosphate cytidyltransferase (GbMECT) gene from *Ginkgo biloba*

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Abstract

2-*C*-methyl-D-erythritol 4-phosphate cytidyltransferase (MECT), the third enzyme of the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, catalyzes formation of 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol from MEP. *GbMECT*, presumably involved in ginkgolide biosynthesis, was cloned and characterized from *Ginkgo biloba* embryonic roots. The protein containing the N-terminal chloroplast transit peptide consisted of 327 amino acid residues. Complementation of *GbMECT* with *Escherichia coli* NMW33, *ygbP* (*EcMECT*) knock-out mutant, rescued the mutant, confirming the function of the protein. Transcription levels of *GbMECT* remained generally constant in embryonic roots and leaves for 1 month. Full 88 N-terminal residues were necessary to deliver the protein into the chloroplast as shown by protein-targeting analysis with GFP as a reporter protein in *Arabidopsis thaliana* protoplasts.

Keywords: *Ginkgo biloba*; Ginkgoaceae; *Arabidopsis thaliana*; Cruciferae; Ginkgolide; 2-*C*-methyl-D-erythritol 4-phosphate cytidyltransferase; Protein targeting

1. Introduction

Ginkgo biloba L. is an important traditional medicinal whose nuts have been used in the Far East as a folk remedy for asthma. In addition, its leaf extract, widely used in modern medicine, contains ginkgolides, a potent platelet-activating factor antagonist (Braquet et al., 1985), as the major medicinal principle.

The biosynthetic pathway from pyruvate (**1**) to ginkgolide (**13**), a diterpene trilactone (see Fig. 1) has attracted the interest of scientists due to its important pharmacological activities (Schwarz and Arigoni, 1999; Nakanishi, 2005). The origin of the isoprenoid units for ginkgolide formation from 2-*C*-methyl-D-erythritol 4-phosphate (**4**) (MEP) pathway was demonstrated via feeding experiments (Schwarz and Arigoni, 1999) (Fig. 1). Our group and a Chinese group subsequently cloned and characterized four MEP pathway genes in *G. biloba*, *GbDXS1* (Kim et al., 2005, 2006b), *GbDXS2* (Kim et al., 2006b), *GbDXR* (Gong et al., 2005; Kim et al., 2006b), and *GbMECS* (Kim et al., 2006a). Also one of the important genes involved in ginkgolide biosynthesis was reported by Schepmann et al. (2001). These researchers cloned the levopimaradiene synthase (LPS) gene whose encoded protein is responsible for cyclization of geranylgeranyl diphosphate (**11**).

In this study, we have cloned and characterized the gene for MEP cytidyltransferase (MECT), whose encoded protein transforms **4** into 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (**5**), from *G. biloba* (GenBank

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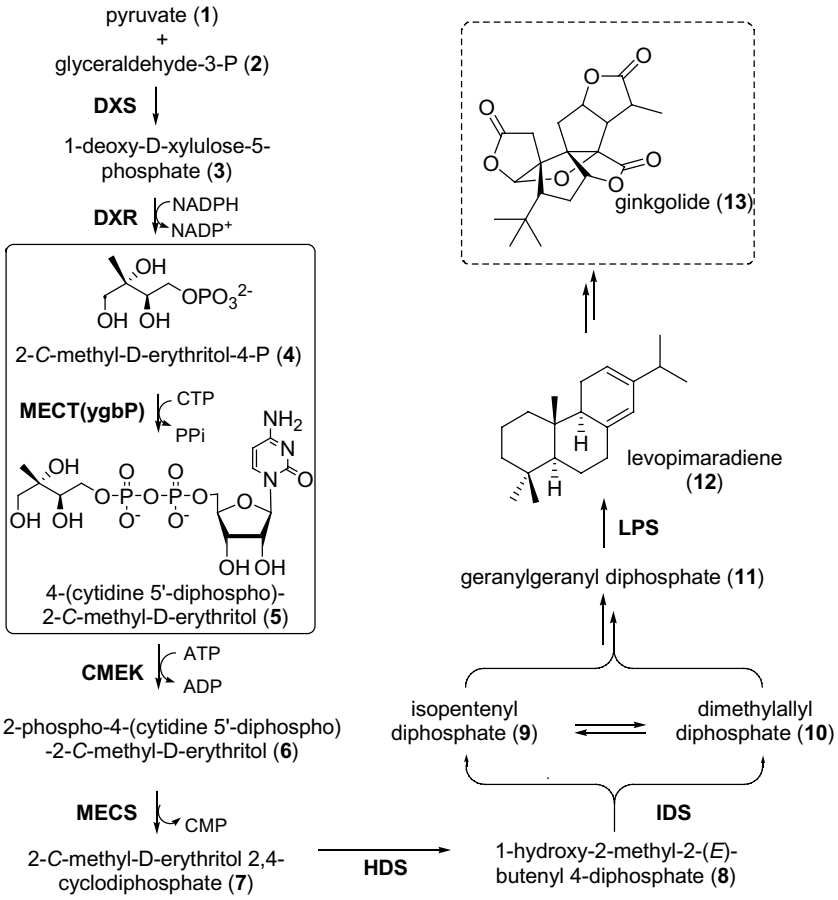

Fig. 1. Ginkgolide biosynthesis through MEP pathway. CMEK, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase; MECS, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase; IDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase.

Accession No. DQ102360). Although several microbial *MECT*s have been isolated and characterized (Kuzuyama et al., 2000; Rohdich et al., 1999), only one report on the functional characterization of *MECT* is available to date in plants (Rohdich et al., 2000). Intracellular targeting of GbMECT and transcription of the gene with respect to ginkgolide biosynthesis are also presented.

2. Results and discussion

2.1. Cloning of GbMECT cDNA and sequence analyses

Initial PCR designed to clone the conserved core region produced a 257-bp fragment; the latter had about 80 and 90% identity and similarity with known *MECT*s through a BlastX search. Based on this sequence, two gene-specific primers were designed and used in the rapid amplification of cDNA end (RACE) PCR. In this way, 5′- and 3′-RACE PCRs, respectively, afforded 747- and 732-bp fragments. A 1411-bp full-length cDNA consisting of a 984-bp open reading frame (ORF) was obtained by combining the sequence information of 3′- and 5′-RACE fragments. At the DNA level, *GbMECT* had 70.7 and 70.2% identities with *Arabidopsis thaliana MECT* (*AtMECT*) and a putative *Oryza sativa MECT* (*OsMECT*). The deduced amino acid sequence of GbMECT consisting of 327 residues had theoretical M_r and p*I* values of 36.3 kDa and 9.08, respectively. The mature protein, cut at A88 based on the alignment result to remove the predicted transit peptide (Fig. 2), had 75.8 and 74.5% identities at the amino acid level with *A. thaliana* and *O. sativa*, respectively. The homology-based 3-D structural model of GbMECT without the transit peptide (data not shown) suggested that the overall structure of GbMECT is consistent with the *Escherichia coli* ygbP crystal structure (Richard et al., 2001). GbMECT had seven α-helices and ten β-sheets connected by turns and loops, and Lys124 and Lys310 were predicted to contribute to the stabilization of the pentacoordinate transition state of the phosphate through coordination with Mg^{2+}.

2.2. Molecular evolution analysis

A phylogenetic tree was constructed using the deduced amino acid sequences of MECTs, putative or functionally confirmed, from plants and microorganisms (Fig. 3). Although sequence data of plant origin were limited compared to those of microorganisms, it was clear that

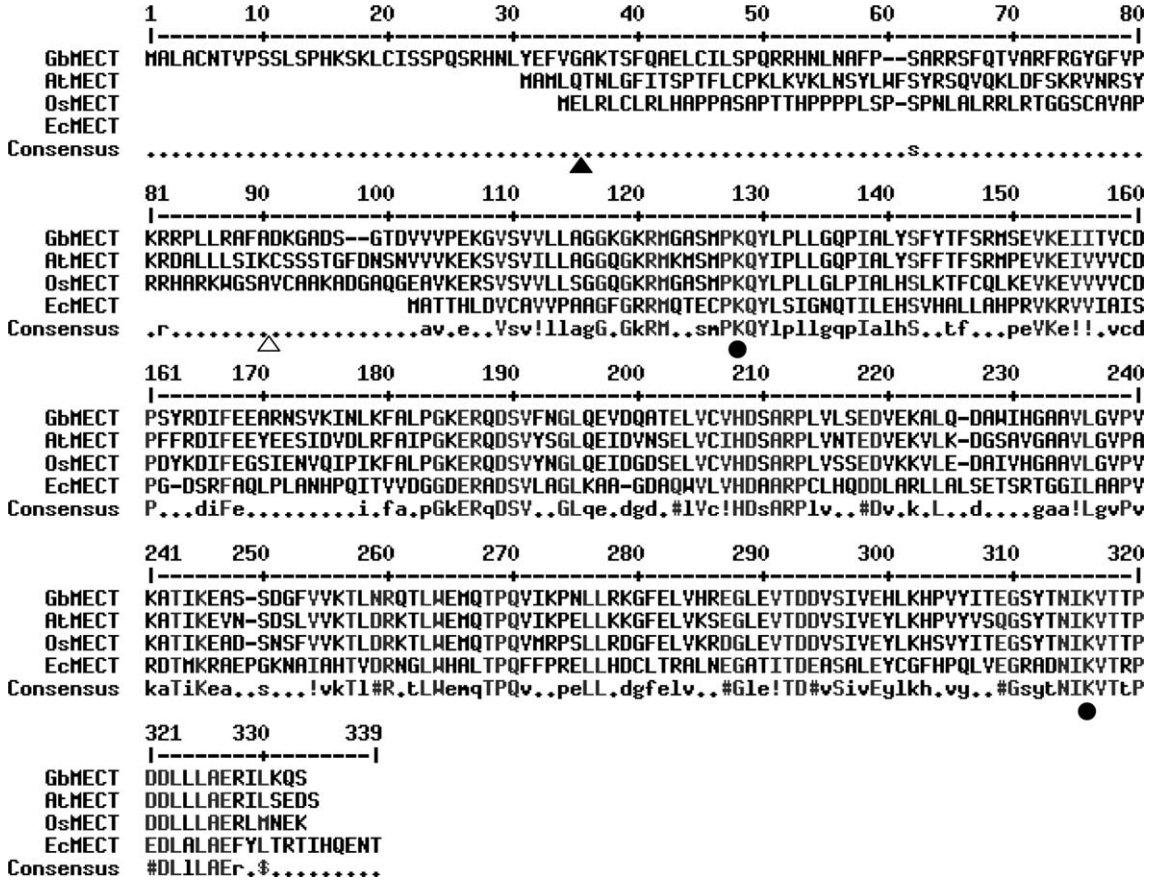

Fig. 2. Amino acid alignment of plant origin MECTs: GbMECT (*G. biloba*), AtMECT (*A. thaliana*), OsMECT (*O. sativa*). *E. coli* MECT (ygbP) is shown for reference. Transit peptide cleavage site predicted by ChloroP and TargetP programs is marked with a black triangle, while the white triangle indicates the putative cleavage site deduced from the cleavage site of AtMECT and OsMECT. The magnesium ion and CTP-binding site are marked with a circle.

GbMECT belonged to the plant group. GbMECT clustered together with another gymnosperm MECT (PtMECT), and thus could be distinguished from angiosperm MECTs. GbMECT and PtMECT formed a broader clade with monocot plants such as *Zea mays* and *O. sativa*, which in turn formed a separate clade from dicots such as *Glycine max* and *A. thaliana*. Other MEP pathway genes from *G. biloba*, *GbDXS*, *GbDXR* and *GbMECS*, have also been reported to form clades separate from those of angiosperms (Kim et al., 2006a,b), although without a broader clade formation with the monocots. Confirmation of this observation, based on the limited data set, could be made when more sequence data of plant MECTs become available.

2.3. Functional identification of GbMECT

Escherichia coli strain NMW33, with a defect ygbP (EcMECT) caused by the mutation of L120F, was used for functional identification of GbMECT. NMW33 had an extra plasmid pTMV20KM carrying the last three mevalonate pathway genes. The plasmid allowed growth on the LB medium only in the presence of mevalonate (Fig. 4A and B). Transformation of NMW33 with GbMECT(Δ35)-pMW plasmid could rescue NMW33 when grown in the absence of mevalonate (Fig. 4C),

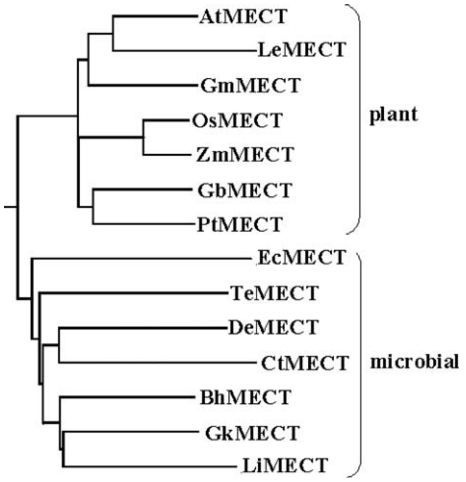

Fig. 3. Phylogenetic tree of MECTs. Gymnosperm GbMECT (*G. biloba*) and PtMECT (*P. taeda*) were separated from angiosperm MECTs. Plant origin: GbMECT (*G. biloba*, Genbank Accession No. DQ102360), AtMECT (*A. thaliana*; AK118110), OsMECT (*O. sativa*; XM_463571), GmMECT (*G. max*; BI321360), ZmMECT (*Z. mays*; DR800609), LeMECT (*Lycopersicon esculentum*; AW934532), PtMECT (*P. taeda*; DR692295). Microbial origin: CpMECT (*Chlamydophila pneumoniae* AR39; NC_002179), BhMECT (*Bacillus halodurans* C-125; BA000004), LiMECT (*Listeria innocua* Clip11262; NC_003212), GkMECT (*Geobacillus kaustophilus* HTA426; NC_006510), TeMECT (*Thermosynechococcus elongatus* BP-1; BA000039), DeMECT (*Dehalococcoides ethenogenes* 195; NC_002936), CtMECT (*Clostridium tetani* E88; NC_004557).

indicating that GbMECT was functionally active. Plasmid pMNMW33, which carried *E. coli ygbP*, was used as a positive control.

2.4. Transcription level of GbMECT in embryo tissue

About 10 *G. biloba* embryos grown in the hormone-free MS medium were harvested every week for 4 weeks. Real-time PCR data showed that the *GbMECT* transcription levels of the embryo were maintained relatively constant at a comparable level both in the roots and leaves throughout the sampling period (data not shown). Transcripts of *GbDXS2* and *LPS*, the genes specific for ginkgolide biosynthesis, occurred exclusively in roots, where ginkgolide biosynthesis takes place. However, *GbDXS1* (Kim et al., 2006b), putatively performing a household function, was transcribed both in the roots and, at a higher level, in the leaves. Transcripts of *GbDXR* (Kim et al., 2006b) and *GbMECS* (Kim et al., 2006a), being single-copy genes, were found in both tissue types. The pattern of the *GbMECT* transcription level did not follow those of *GbDXR* and *GbMECS*.

2.5. Analysis of transit sequence and protein targeting in Arabidopsis protoplast

All plant MEP pathway genes are known to have an N-terminal chloroplast transit peptide sequence that enables importation of proteins from the cytosol into the chloroplast. Vigorous demonstrations on the target location in the cell have been established for LeDXS (Lois et al., 2000), AtDXR (Carretero-Paulet et al., 2002), AtHDS

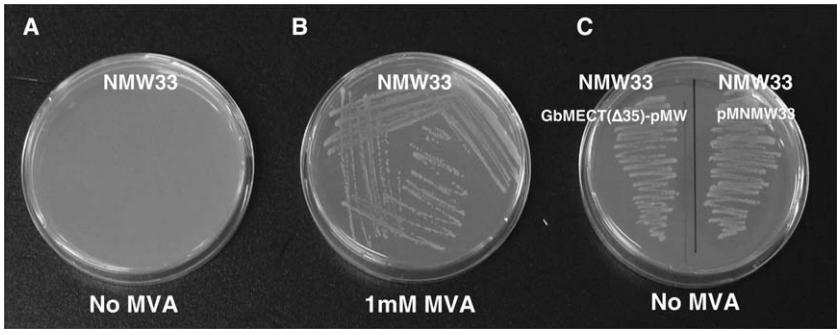

Fig. 4. Complementation assay with *E. coli ygbP* knock-out mutant. NMW33 containing mevalonate cluster genes showed conditional growth: (A) without mevalonate (MVA); (B) with mevalonate. Plasmid GbMECT(Δ35)-pMW rescued the mutant grown on medium without mevalonate, indicating that GbMECT had the expected activity (C). pMNMW33 harboring intact *E. coli ygbP* was used as the positive control.

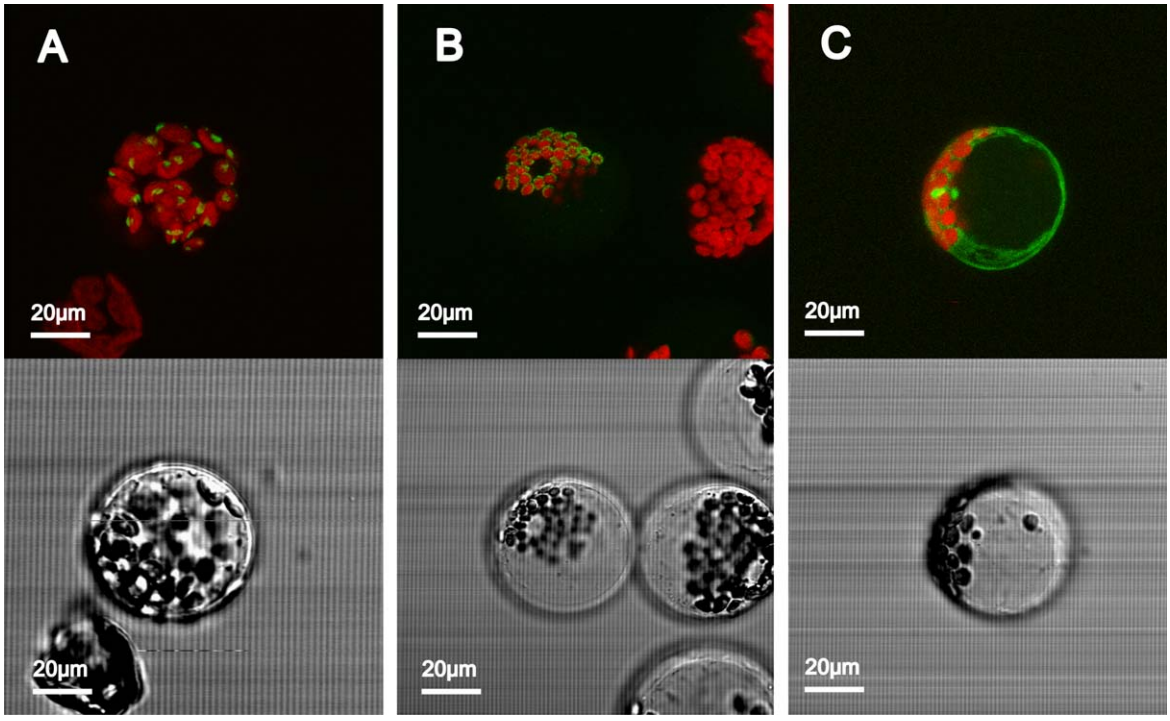

Fig. 5. Targeting of GbMECT-GFP into *Arabidopsis* protoplast. (A) Full ORF-smGFP construct; (B) GbMECT-TE1-smGFP (for Δ35); (C) GbMECT-TE2-smGFP (for Δ88). Upper and lower panels show merged and bright-field images, respectively.

(Querol et al., 2002), and GbMECS (Kim et al., 2006a). Four transit sequence-predicting programs, Predator, ChloroP, PSORT, and TargetP, were used to analyze the N-terminal sequences of selected seven plant origin MECTs. ChloroP predicted that all MECTs are targeted to the chloroplast, whereas other programs assigned the target of some plant MECT to mitochondria, cytoplasm or endoplasmic reticulum (data not shown). GbMECT had the longest N-terminal sequence among the known plant MECTs when compared to that of *E. coli*. ChroroP and TargetP predicted that GbMECT has a relatively short transit peptide consisting of 35 residues. However, comparison of the sequence with those of AtMECT and OsMECT predicted A88 as a cleavage position for GbMECT (open triangle, Fig. 2).

To resolve the discrepancies among predictions, two GbMECT subclones with the sites of cleavage set at G35 (Δ35) and A88 (Δ88) were constructed. The full-length GbMECT ORF and the truncated GbMECTs fused to smGFP were transiently expressed in the *A. thaliana* protoplast. In the case of ORF-smGFP, green fluorescence was detected inside the chloroplast, whereas the Δ35-smGFP showed a chloroplast envelope-associated fluorescence (Fig. 5). Cytosolic expression was evident for Δ88-smGFP. This suggested that the N-terminal sequence from A35 through A88 was necessary to recruit the immature GbMECT to the membrane, but was not sufficient to deliver the protein into the chloroplast across the envelope. Fluorescence of ORF-smGFP did not coincide with the chloroplast autofluorescence, suggesting an association with thylakoid (Bauer et al., 2004). This interpretation on the suborganellar location of GbMECT should be substantiated by further experiment. Taken together, these data clearly showed that GbMECT had an N-terminal chloroplast transit peptide of 88 residue-long, and was transported into the chloroplast with the suborganellar location suggested to be thylakoid.

3. Conclusions

We identified and characterized a MEP cytidyltransferase (GbMECT) gene from *G. biloba* consisting of 984-bp corresponding to a protein encoding 327 amino acids. The functional activity of GbMECT was confirmed via a complementation assay with the *E. coli* knock-out mutant, NMW33. GbMECT shared the same active site motif conserved in Lys124 and Lys310. The phylogenetic tree, constructed with microbial and plant origin MECTs, revealed that GbMECT and PtMECT form a gymnosperm clade that is distinguishable from the angiosperm clade. *GbMECT* was maintained at the same transcription level in both roots and leaves during the 1-month period of embryo culture. The full-length GbMECT-smGFP transiently expressed in *A. thaliana* protoplast was found in the chloroplast, indicating an association with thylakoid. The full 88 N-terminal residues were necessary for correct transportation into the chloroplast as opposed to the prediction that a 35 N-terminal residue is the transit peptide.

4. Experimental

4.1. Plant materials

Ginkgo nuts were purchased from Nammun Market, Suwon, Korea. After dehulling and sterilization with 4% NaOCl solution for 20 min, the seeds were washed extensively with distilled H_2O, and the cotyledon was removed from the seeds. The embryo was placed on hormone-free MS medium and incubated at 23 °C under 16 h/8 h light/dark regimen.

4.2. RNA isolation and cDNA synthesis

Cetyltrimethylammonium bromide (CTAB) method was used isolation of total RNA from a 1-month-old embryo culture (Chang et al., 1993). Messenger RNA was prepared from total RNA using PolyATract mRNA Isolation System (Promega). Single strand cDNA was synthesized using the GeneRacer kit (Invitrogen) according to the manufacturer's protocol. For real-time PCR, total RNA (2 μg) was reverse-transcribed according to the manufacturer's protocol (Qiagen) using an oligo$(dT)_{17}$ primer. The resultant single strand cDNA mixtures were used as templates for real-time PCR.

4.3. Cloning of GbMECT full-length cDNA by RACE

The primer pair of MECT-F4 (5′-CCAGGCAAAGAGAGACAAGA-3′) and MECT-B5 (5′-TGTGGTGTATGCATTTCCCA-3′) designed through the conserved sequences of the known *MECT*s was used for PCR to clone the core cDNA fragment. All PCRs were initiated with the hot start method (D'Aquila et al., 1991) using cDNA template and Takara Ex-Taq polymerase. The PCR product was cloned into the pGEM-T Easy vector (Promega) and sequenced. New pairs of primers for RACE were designed based on sequence information. The primer pair of Generacer 5′-nested (5′-GGACACTGACATGGACTGAAGGAGTA-3′) and MECT-5B (5′-TTTGCCTGTTTAATGTCTTCACCAC-3′) was used for 5′-RACE, while the primer pair of Generacer 3′-nested (5′-CGCTACGTAACGGCATGACAGTG-3′) and MECT-3F (5′-GGAAGTTGACCAAGCTACAGAACTTG-3′) was used for 3′-RACE. Each RACE product was cloned and sequenced as mentioned above. Full-length ORF cDNA was amplified with MECT-TE-START (5′-GGATCCAAATGGCATTAGCATGTAACACC-3′) and MECT-TE-STOP (5′-GGATCCCAACTTTGTTTCAAAATCCT-3′) primer pair and cloned into smGFP vector with *Bam*HI site for protoplast targeting (GbMECT-GFP plasmid). MECT-pMW-START (5′-GGATCCTCCAAAACATCCTTCCAA-3′) and MECT-TE2-START

(5′-GGATCCAGATAAGGGTGCTGATTCT-3′) primers were amplified with MECT-TE-STOP primer to delete the N-terminal transit peptide (GbMECT-TE1-GFP plasmid for Δ35 and GbMECT-TE2-GFP plasmid for Δ88 plasmids). For the complementation assay, ORF cDNA devoid of putative 35 residue-long chloroplast transit peptide sequence was amplified with MECT-pMW-START and MECT-pMW-STOP (5′-GGATCCTTCAACTTTGTTTCAAAATCC-3′) primer pair and cloned into pMW118 vector (Nippon Gene) with *Bam*HI site to produce GbMECT(Δ35)-pMW plasmid.

4.4. Bioinformatic analyses and protein modeling

The multalin program (http://prodes.toulouse.inra.fr/multalin/multalin.html) was applied for the alignment of plant MECT amino acids. The putative target location of plant MECTs were predicted online with ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), Predator (http://genoplante-info.infobiogen.fr/predotar/), TargetP (http://www.cbs.dtu.dk/services/TargetP/), and PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) programs, respectively. A phylogenetic tree was constructed using the ClustalW program at default setting, and the theoretical molecular weight and p*I* value were calculated using the Compute p*I*/M_w tool (http://ca.expasy.org/tools/pi_tool.html). Homology-based structural modeling was carried out through Swiss-Model (http://swissmodel.expasy.org/) using the *E. coli* MECT structure (Richard et al., 2001) as the template.

4.5. Complementation assay

Escherichia coli ygbP mutant, NMW33, which harbored a pTMV20KM plasmid containing mevalonate kinase, phosphomevalonate kinase, and pyrophosphomevalonate decarboxylase genes from *Streptomyces* sp. CL190, was maintained on LB medium supplemented with 1 mM mevalonate and 50 μg/ml kanamycin (Kuzuyama et al., 2000). NMW33 was transformed with GbMECT(Δ35)-pMW plasmid and selected on LB medium without mevalonate.

4.6. Real-time PCR

For quantification of *GbMECT* transcripts in the *G. biloba* embryo culture, quantitative real-time PCR was carried out with the RTMECT-F (5′-CAAGCTACAGAACTTGTATG-3′) and RTMECT-B (5′-CACAAAGCCATCACTACT-3′) primer pair. The reaction was carried out in triplicate for 45 cycles on a Rotor-Gene 2000 Real Time Amplification System (Corbett Research) using a Qiagen Quantitect SYBR Green PCR system. The PCR reaction (50 μl) consisted of 1× master mix, 0.5 μM each primer, and 100 ng cDNA as the template. cDNAs for quantification were prepared from about 10 plants. The PCR amplification was performed under the following conditions: 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 45 °C for 30 s, and 72 °C for 30 s. For quantification standard, the PCR product amplified from cDNA was isolated, and the concentration of the product was measured at 260 nm to calculate the number of cDNA copies in the sample as described by Yin et al. (2001). The range of cDNA concentrations in standardization reactions was 10^4–10^8 copies/μl.

4.7. Protein targeting

Protoplasts were isolated and transfected with GbMECT-GFP, GbMECT-TE1-GFP, and GbMECT-TE2-GFP plasmids using a modified polyethylene glycol method as described for *A. thaliana* (Abel and Theologis, 1994). Three hundred microliters of the protoplast suspension (10^6/ml) was transfected with 20 μg each plasmid DNA (1 μg/μl), and the transfected protoplasts were incubated at 22 °C in darkness. After transformation, expression of the fusion protein was monitored, and the images were captured with MRC-1024 Confocal Laser Scanning Microscope system (Bio-Rad). Data were then processed using CAS program (Bio-Rad) and Adobe Photoshop v7.0 software.

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