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GENETICS AND GENOMICS

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Cloning and characterization of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECS) gene from *Ginkgo biloba*

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Abstract *Ginkgo biloba* contains secondary metabolites with interesting pharmacological properties, including highly modified diterpenoid ginkgolide, potent and selective antagonist of platelet-activating factor. 2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate synthase gene (*GbMECS*) involved in ginkgolide biosynthesis pathway was cloned and characterized from *G. biloba* embryonic roots, and the full open reading frame was deduced as protein consisting of 238 amino acid residues. Putative mature protein with a 179 residue-long sequence, obtained by deleting N-terminal chloroplast transit peptide region composed of 59 amino acid residues, rescued *Escherichia coli* NMW26, an *E. coli* knock-out mutant of *ygbB* (*EcMECS*). Transcription levels of *GbMECS* were two-fold higher in embryo roots compared to leaves. When full-length *GbMECS* with chloroplast transit peptide sequence was fused to green fluorescent protein gene (*GFP*), and transiently expressed in *Arabidopsis thaliana* protoplast, green fluorescence was found in chloroplast, indication of protein transportation into plastid.

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Introduction

Ginkgo biloba, a living fossil tree, has been used as a traditional medicine in Far Eastern countries including Korea, and, in recent years, its leaf extract has been widely sold as phytomedicine in Europe as well as in Korea, and as a dietary supplement worldwide. One of the most active ingredients of the extract beside flavonoids is diterpene trilactones (TTLs), which contains ginkgolides A, B, C, J, and M, and bilobalide (Strømgaard and Nakanishi 2004), ginkgolides being potent antagonists of the platelet-activating factor receptors (Braquet et al. 1985) and selective antagonists of glycine receptors (van Beek 2005). Bilobalide is another predominant ginkgo TTL, which exhibits neuroprotective actions by decreasing the release of excitotoxic amino acids, particularly glutamate and aspartate (Huang et al. 2003).

Despite the importance of TTLs in human health, only a few reports have been published on the genes involved in ginkgolide biosynthetic pathway, including levopimaradiene synthase (LPS) gene, which catalyzes the first committed step of geranylgeranyl diphosphate cyclization (Schepmann et al. 2001), and geranylgeranyl diphosphate synthase gene (Liao et al. 2004). The authors and a Chinese group reported several genes in methylerythritol 4-phosphate (MEP) pathway, the earlier step of TTL biosynthesis (Kim et al. 2005, in press; Gong et al. 2005). MEP pathway, which operates in eubacteria and plant plastids, converts pyruvate and glyceraldehyde 3-phosphate into common terpene precursors, isopentenyl diphosphate and dimethylallyl diphosphate. Simply put, the pathway operates in plants for mono-, di-, and tetraterpene biosyntheses, whereas mevalonate pathway for sesqui- and triterpenes. However, cross-talk that allows flow of isopentenyl diphosphate and dimethylallyl diphosphate between MEP and

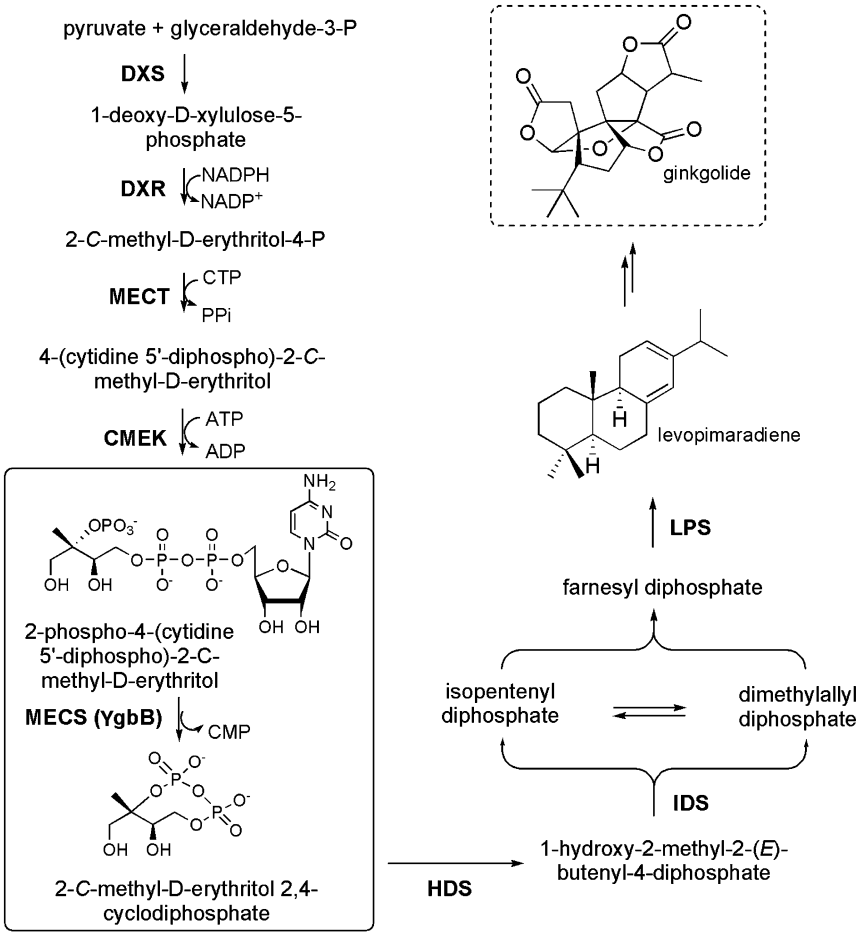

Fig. 1 Ginkgolide biosynthesis pathway. HDS 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase, IDS 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase. For other abbreviations, refer to the text

mevalonate pathways has been reported (Hemmerlin et al. 2003). Therefore, ginkgo TTLs, being diterpenoids, would have originated from MEP pathway (Kim et al. in press). Among the seven enzymic transformations expected to function in ginkgo MEP pathway, condensation of pyruvate and glyceradehyde 3-phosphate into 1-deoxy-D-xylulose 5-phosphate is catalyzed by two classes of 1-deoxy-D-xylulose 5-phosphate synthase, GbDXS1, which is transcribed both in ginkgo leaves and roots, and GbDXS2, which is specific in roots, where ginkglides are biosynthesized (Kim et al. 2005, in press). DXP is then converted into MEP in the first committed step of the pathway by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), whose gene is transcribed both in the leaves and the roots (Kim et al. in press; Gong et al. 2005). The ensuing steps are then serially catalyzed by MEP cytidyl transferase (MECT) and 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMEK), yet to be described in ginkgo (Fig. 1).

In this study, we cloned and characterized 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (EC:4.6.1.12) from *G. biloba* (GbMECS, GenBank accession number AY494187), the fifth enzyme in the MEP pathway sequence, transforming 2-phospho-4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol into 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (Fig. 1). Intracellular targeting of GbMECS and transcription of the gene with respect to ginkgolide biosynthesis were also evaluated.

Materials and methods

Plant materials

Following extensive washing in distilled water, ginkgo seeds were sterilized with 4% NaOCl solution for 20 min, and the cotyledons were removed from the seeds. The embryos were then placed in hormone-free MS medium and incubated at 23 °C under 16 h/8 h light and dark regimen. The seedlings were harvested every week.

RNA isolation and cDNA synthesis

Total RNA was isolated from a 1-month-old embryo culture using the CTAB method (Chang et al. 1993). Messenger RNA was prepared from total RNA using PolyATract mRNA Isolation System (Promega, http://www.promega.com). GeneRacer Kit (Invitrogen, http://www.invitrogen.com) was used to synthesize single strand cDNAs following the manufacturer's protocol. For real-time polymerase chain reaction (RT-PCR), 2 μg of total RNA was reverse-transcribed according to the manufacturer's protocol (Qiagen, http://www1.qiagen.com) using an oligo(dT)$_{17}$ primer. The resulting single strand cDNA mixtures were used as templates for RT-PCR.

Cloning of *GbMECS* full-length cDNA

The primer pair of YB-2 (5′-GAYMGNGGNTGYGAR GC-3′) and YB-4 (5′-TANCCNGCYTCRTSCAT-3′) designed from the conserved sequences of the known bacterial and plant *MECS*s was used for the PCR to clone the core cDNA fragment. All PCRs were initiated with hot start method using the single strand cDNA template and Ex-Taq polymerase (Takara, http://www.takara.com). The PCR product was subcloned into pGEM-T Easy vector (Promega) and sequenced. New pairs of primers were designed for rapid amplification of cDNA end (RACE) based on the sequence information. The primer pair of GeneRacer 5′-nested (5′-GGACACTG ACATGGACTGAAGGAGTA-3′) and MEC-F (5′-GCT GCTCCACGCCATTTGGGA-3′) was used for the 5′-RACE PCR reaction, while that of GeneRacer 3′-nested (5′-CGCTACGTAACGGCATGACAGTG-3′) and MEC-B (5′-GATGGTGATGTGCTGCTTCAT-3′) was used for the 3′-RACE PCR reaction. Each RACE product was cloned as mentioned above. Full-length open reading frame (ORF) cDNA was amplified with MECS-TE-START (5′-GGATCCAAATGAGCTGCCGCA-3′) and MECS-TE-STOP (5′-GGATCCCACTTCTTCATCAAAAGTACA-3′) primer pair, and cloned into smGFP vector with BamHI site to obtain GbMECS-GFP plasmid for protoplast-targeting experiment. ORF cDNA, from which the predicted chloroplast transit peptide sequence was removed, was amplified with MECS-pMW-START (5′-GGATCCTCAATCTGCCAATCCAGC-3′) and MECS–STOP-B (5′-CGCGGATCCTCACTTCTTCATCAAA-3′) primer pair, and cloned into pMW118 vector with BamHI site (Nippon Gene, http://www.nippongene.com) to construct MECS-pMW plasmid for functional analysis.

Bioinformatic analyses and protein modeling

Multalin program (http://prodes.toulouse.inra.fr/multalin/) was applied for the alignment of plant MECS amino acid. Prediction of chloroplast-targeting peptides and construction of phylogenetic tree were preformed, respectively, using ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), and ClustalW programs at default setting. Theoretical molecular weight and p*I* value were calculated using Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). Homology-based structural modeling was carried out through Swiss-Model (Schwede et al. 2003) and DeepView (Swiss Pdb-viewer) program (http://swissmodel.expasy.org/) using *E. coli* MECS structure (Kishida et al. 2003) as the template.

Complementation assay

NMW26, an *E. coli ygbB* (*EcMECS*) mutant harboring the pTMV20km plasmid, which carries mevalonate kinase, phosphomevalonate kinase, and pyrophosphomevalonate decarboxylase gene from *Streptomyces sp*. CL190, was maintained on LB medium supplemented with 1 mM potassium mevalonate and 50 μg/mL kanamycin (Takagi et al. 2000). NMW26 was transformed with MECS-pMW and selected on LB medium without mevalonate. Mevalonate was generated by reacting equal volumes of 2 M each mevalonolactone and potassium hydroxide at 37 °C for 30 min.

Transcription level analysis by RT-PCR

The reaction was carried out in triplicate for 45 cycles on Rotor-Gene 2000 Real Time Amplification System (Corbett Research, http://www.corbettresearch.com) using Qiagen Quantitect SYBR Green PCR system. PCR was performed with 50 μL reaction consisting 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. 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 its concentration 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.

Arabidopsis protoplast transient expression assay

Protoplasts were isolated and transfected with *GbMECS-GFP* plasmid using a modified polyethylene glycol method as described for *A. thaliana* (Abel and Theologis 1994). Three hundred microliters of protoplast suspension (10^6/mL) was transfected with 20 μg of *GbMECS-GFP* 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, http://www.bio-rad.com). Data were then processed using CAS program (Bio-Rad) and Adobe Photoshop v7.0 software.

Results and discussion

Cloning of *GbMECS* cDNA and sequence analyses

Initial PCR designed for the conserved sequence produced a 186-bp fragment, which had the expected nucleotide sequence as revealed through BlastX search. Based on this sequence, two gene-specific primers were designed and used in 5′- and 3′-RACE PCRs, respectively obtaining 531- and 565-bp fragments. From the 984-bp long full sequence obtained by combining the sequence information of 3′- and 5′-RACE fragments, full-length *GbMECS* cDNA sequence

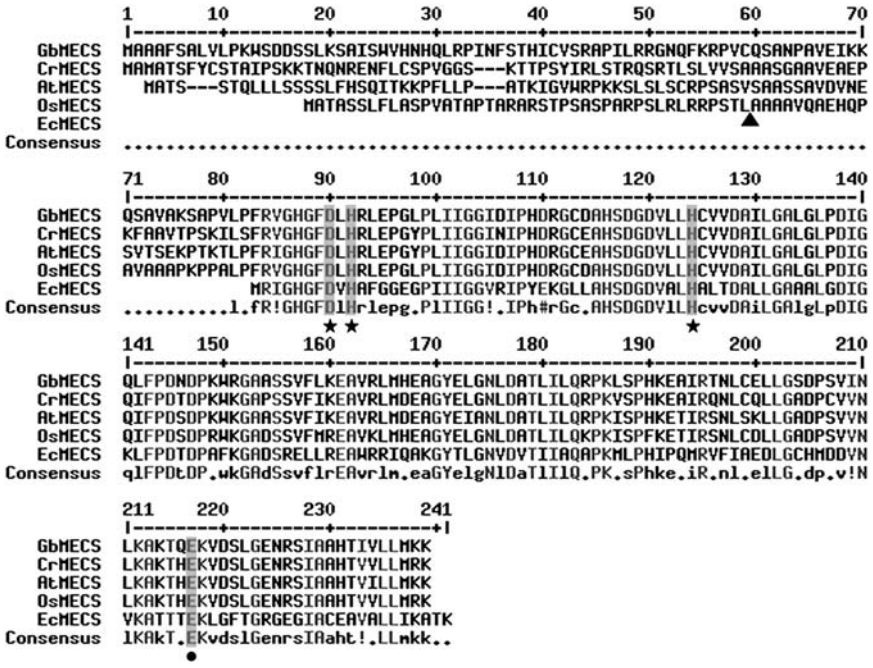

Fig. 2 Amino acid alignment of plant origin MECSs. GbMECS *G. biloba*, CrMECS *Catharanthus roseus*, AtMECS *A. thaliana*, OsMECS *O. sativa*. For reference *E. coli* MECS (YgbB or EcMECS) is shown. Putative cleavage site of plastid transit peptide is labeled with triangle. *Stars* indicate amino acids coordinating with zinc ion. Magnesium or manganese ion binding site is marked with a *circle*

of 935 bp, consisting of 717-bp long ORF and 3′- and 5′-untranslated regions, was secured. The deduced protein consisted of 238 amino acid residues. ChloroP program predicted that the deduced GbMECS has the N-terminal chloroplast transit peptide consisting of 59 residues, and plastid transit peptides of the putative MECSs from other plants such as *Catharanthus roseus* (*CrMECS*), *Oryza sativa*, (*OsMECS*), and *A. thaliana* (*AtMECS*) were predicted to have similar lengths (Fig. 2). In the case of *E. coli* MECS, this putative transit peptide and additional 23 residues linking the mature sequence are absent. These results indicate that the deduced putative mature GbMECS, devoid of transit peptide region, consists of 179 residues, with theoretical molecular weight and p*I* of 19.3 kDa and 6.47, respectively. *GbMECS* had about 70–78% sequence identities with putative *CrMECS*, *AtMECS*, and *OsMECS*, when the predicted transit sequences were excluded. On the other hand, sequence identities among the corresponding putative mature proteins were higher, ranging 81–86%.

Tertiary and homotrimeric quaternary structures of *E. coli* YgbB were elucidated by crystallography (Kishida et al. 2003). The homology-based 3-D structure model of GbMECS without the transit peptide sequence showed that overall structure of GbMECS, which contained seven β-sheets, two θ-helices, and four α-helices, has high similarity with the known EcMECS structures, except for one extra β-sheet (data not shown). The active site residues of EcMECS, such as D8, H10, and H42 (Zn^{2+} ligands), and E135 (Mg^{2+} or Mn^{2+}-binding site) were conserved in GbMECS (Fig. 2). In addition, zinc and magnesium ions are known to play important roles in maintaining precise geometry of CDP moiety during catalysis (Steinbacher et al. 2002). These findings therefore suggest that GbMECS shares the same active site motif with *E. coli* YgbB.

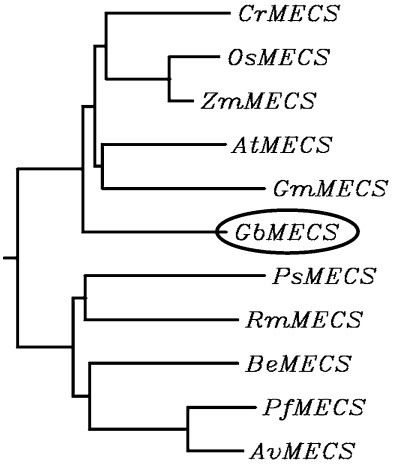

Fig. 3 Phylogenetic tree of MECSs. Gymnosperm GbMECS (*Ginkgo biloba*, AY494187) was separated from angiosperm MECSs. Plant origin: *CrMECS Catharanthus roseus* (AF250236), *AtMECS Arabidopsis thaliana* (AY085564), *OsMECS Oryza sativa* (AP005823), *GmMECS Glycine max* (AW202270), *ZmMECS Zea mays* (AI712133). Microbial origin: *PsMECT Polaromonas sp.* JS666 (ZP_00362508), *RmMECT Ralstonia metallidurans* CH34 (ZP_00277083), *BcMECT Bacillus cereus* ATCC 10987 (NP_976414), *AvMECS Azotobacter vinelandii* (ZP_00091526), *PfMECS Pseudomonas fluorescens* PfO-1 (ZP_00266490)

Molecular evolution analysis

A phylogenetic tree was constructed using the deduced amino acid sequences of all known putative MECSs from plants and microorganisms (Fig. 3); the sequence data of plant origin were scarce compared to those of microorganisms. The tree clearly showed that GbMECS belongs to the plant group. Despite the insufficient amount of sequence data available, gymnosperm GbMECS was distinctively separated from the angiosperm MECS. Monocot plants

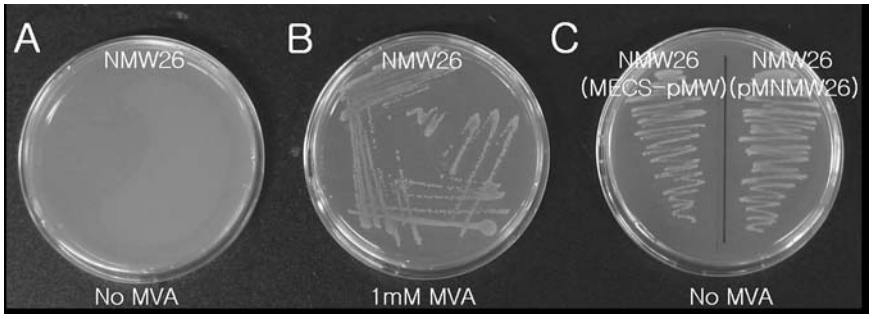

Fig. 4 Complementation assay with *E.coli ygbB* knock-out mutant. NMW26 containing mevalonate cluster genes showed the conditional growth **A** without mevalonate, **B** with mevalonate. **C** Plasmid MECS-pMW rescued the mutant grown on medium without mevalonate, indicating that GbMECS had the expected activity. pMNMW26 harboring intact *E.coli ygbB* was used as the positive control gene

such as *Zea mays* and *O. sativa* were grouped separately into a clade different from that of dicots such as *Glycine max* and *A. thaliana*. Other MEP pathway genes from *G. biloba*, GbDXS and GbDXR, also form a clade separate from those of angiosperms (Kim et al. in press).

Complementation analysis

No activities of plant origin MECS have yet been demonstrated, although several of their sequences including that of the putative CrMECS have been published (Veau et al. 2000). Recently, Burlat et al. (2004) examined tissue-specific transcription pattern of the putative CrMECS showing co-localization of this gene and geraniol 10-hydroxylase gene, which is involved in the first committed step of monoterpene iridoid biosynthesis. To ascertain the catalytic function of the putative GbMECS, *E. coli* strain NMW26, an L120F knock-out mutant on *ygbB*, was used for complementation assay (Takagi et al. 2000). Because NMW26 carried the last three genes of the mevalonate pathway, it could only grow on medium supplemented with mevalonate. Transformation of the knock-out mutant NMW26 with MECS-pMW plasmid enabled the transformant to grow on LB medium without mevalonate supplementation (Fig. 4), demonstrating the catalytic competence of the protein. Introduction of pMNMW26 plasmid carrying the *E. coli ygbB* in pMW118 vector could also rescue the knock-out mutant serving as the positive control.

Transcription level of *GbMECS*

Quantification of the *GbMECS* transcript by real-time PCR showed that the transcription level in the embryo roots was at least two times higher than that in the embryo leaves throughout the one-month period of embryo culture, possibly because the enzyme performed dual role of ginkgolide biosynthesis and vital household function(s) (Fig. 5). Kim et al. (in press) examined transcription levels of *DXS1, DXS2, DXR,* and *LPS* in the embryonic ginkgo leaves and roots. In the case of *GbDXS2* and *LPS*, the transcript occurred exclusively in the roots, where ginkgolide biosynthesis takes place (Cartayrade et al. 1997). However, *GbDXS1*, putatively a household gene, maintained lower level of transcript in the roots than in the leaves at the earlier stage of embryo culture. GbDXR, which is expected to play a dual role of supplying building blocks not only for household metabolism such as carotenoid biosynthesis but also for secondary metabolism exemplified with ginkgolide biosynthesis, maintained 30–50% higher transcription level in the roots than in the leaves. These findings thus suggest that the dual role of MEP pathway enzymes such as GbDXR and GbMECS is reflected by the higher transcription levels of their respective genes in the roots.

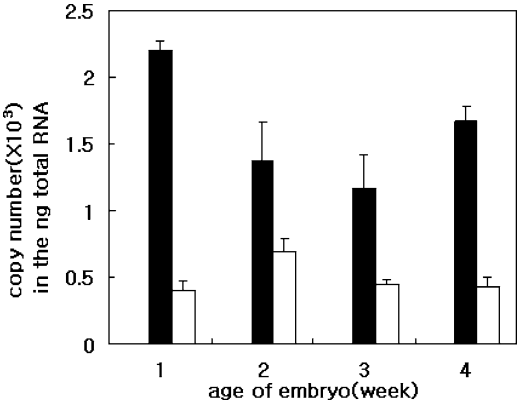

Fig. 5 Changes in transcriptional levels of *GbMECS*. The transcription levels of embryo roots (*black*) were significantly higher compared to those of embryo leaves (*white*)

Protoplast targeting analysis

All MEP pathway genes of plant origin have the N-terminal chloroplast transit peptide sequence, which enables transportation of protein from the cytosol into the chloroplast. GbMECS was also predicted to have the transit peptides consisting of 59 residues, which implies that this protein is also transported into the chloroplast. Therefore, in the ensuing step, GbMECS-GFP fusion protein was constructed to confirm the function of the transit peptide. The fusion protein, transiently expressed in *A. thaliana* protoplast, appeared in the chloroplast as a green color under 500–530 nm

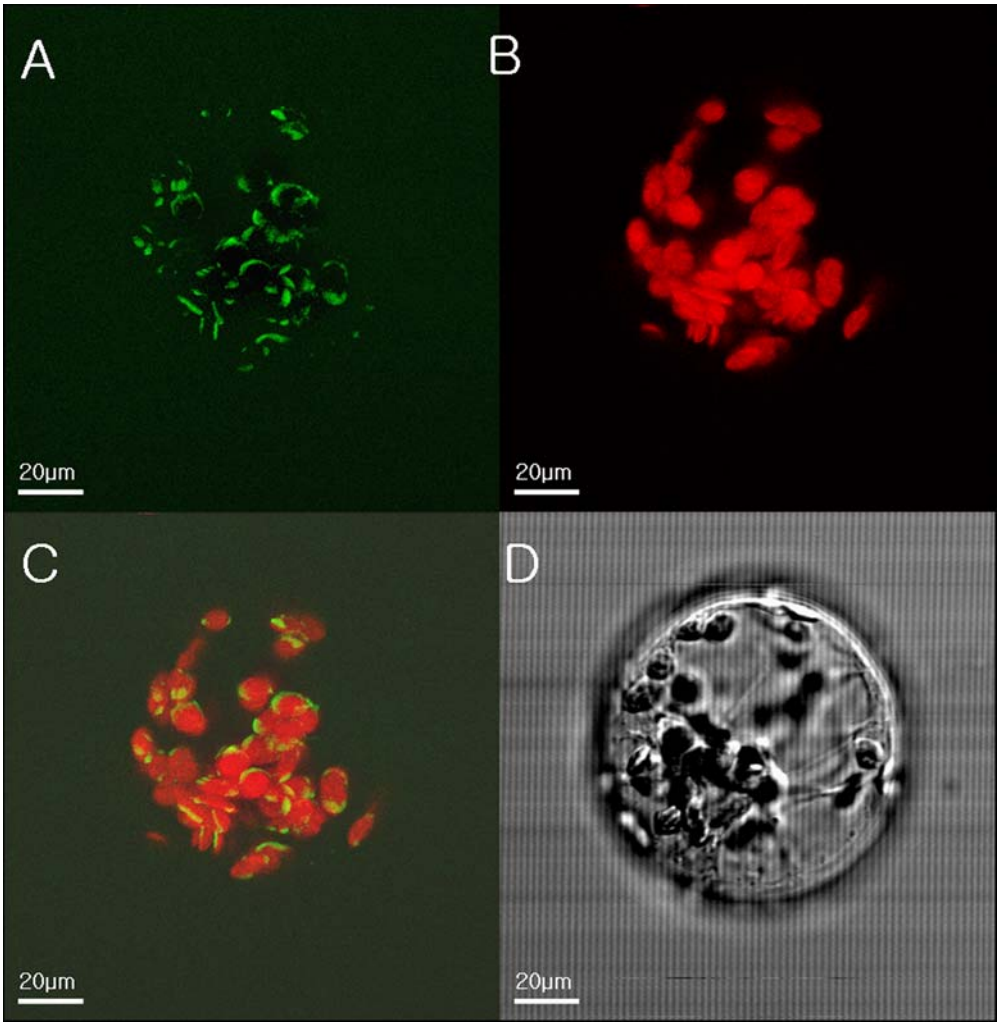

Fig. 6 Targeting of GbMECS-GFP into *Arabidopsis* protoplast. **A** GFP fluorescence. **B** Chlorophyll autofluorescence. **C** Merged image. **D** Bright field image

light (Fig. 6), indicating that it was imported into the chloroplast from the cytosol.

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