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ORIGINAL ARTICLE

Construction and analysis of EST libraries of the *trans*-polyisoprene producing plant, *Eucommia ulmoides* Oliver

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Abstract *Eucommia ulmoides* Oliver is one of a few woody plants capable of producing abundant quantities of *trans*-polyisoprene rubber in their leaves, barks, and seed coats. One cDNA library each was constructed from its outer stem tissue and inner stem tissue. They comprised a total of 27,752 expressed sequence tags (ESTs) representing 10,520 unigenes made up of 4,302 contigs and 6,218 singletons. Homologues of genes coding for rubber particle membrane proteins that participate in the synthesis of high-molecular poly-isoprene in latex were isolated, as well as those encoding known major latex proteins (MLPs). MLPs extensively shared ESTs, indicating their abundant expression during *trans*-polyisoprene rubber biosynthesis. The six mevalonate pathway genes which are implicated in the synthesis of isopentenyl diphosphate (IPP), a starting material of poly-isoprene biosynthesis, were isolated, and their role in IPP biosynthesis was confirmed by functional complementation of suitable yeast mutants. Genes encoding five full-length *trans*-isoprenyl diphosphate synthases were also isolated, and two among those synthesized farnesyl diphosphate from IPP and dimethylallyl diphosphate, an assumed intermediate of rubber biosynthesis. This study should provide a valuable resource for further studies of rubber synthesis in *E. ulmoides*.

Keywords *Eucommia ulmoides* Oliv. · EST · Hardy rubber tree · Isoprenoid · Prenyltransferase · *Trans*-polyisoprene

Abbreviations

DMAPP	Dimethylallyl diphosphate
DXP	Deoxy-xylulose phosphate
FPP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
IPP	Isopentenyl diphosphate
IT	Inner stem tissue
MEP	Methylerythritol phosphate
MLP	Major latex protein
OT	Outer stem tissue
REF	Rubber elongation factor
RPMP	Rubber particle membrane protein

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SRPP	Small rubber particle protein
TIDS	*Trans*-isoprenyl diphosphate synthase

Introduction

Natural rubbers are some of the most important chemical products widely utilized in modern society. Although the rubbers are essential resources for the chemical industry with significant economic benefits, their biosynthetic mechanisms are yet to be completely elucidated. Rubbers consist entirely of high-molecule polyisoprene which can be subclassified into either *trans*- or *cis*-polyisoprene. Hardy rubber tree, *Eucommia ulmoides* is one of the best known *trans*-polyisoprene rubber producers. It is a dioecious diploid ($2n = 34$ and 1C = 0.74 pg; 725 Mbp) plant, native to China and the only species placed in the family *Eucommiaceae* (euasterids I order Garryales) (Hu 1979; Hanson et al. 2001; The angiosperm phylogeny group 2003). Sister species of the same genus are found only as fossil remains (Call and Dilcher 1997). *E. ulmoides* is extensively cultivated in China principally for its bark as a source of an herbal drug of traditional Chinese medicine (Hu 1979; Pang et al. 2008). The cells in the cortex, pith, and secondary phloem of the stem, as well as those in the parenchyma surrounding vascular bundle of the leaf, contain abundant high-molecular-weight *trans*-polyisoprene rubbers (Weiss 1891). These are referred to as nonarticulated unbranched laticifers (Evert 2006), which often develop very thick cell walls and resemble fibers in distribution. The accumulation of *trans*-polyisoprene rubbers in *E. ulmoides* pericarps can reach almost 30 % of dry weight (Nakazawa et al. 2009), with average molecular weights of about 330,000 in the bark and 220,000 in leaves (Hendricks et al. 1945; Tangpakdee et al. 1997; Nakazawa et al. 2009).

Trans-polyisoprene rubbers show less flexibility and poorer low-temperature thermoplasticity ($T_m = 54.5$ °C) (Kent and Swinney 1966; Enoki et al. 2003; Rose and Steinbüchel 2005) than *cis*-polyisoprene rubbers. Whereas *cis*-polyisoprene rubbers have been found in about 2,500 plant species, including *Hevea brasiliensis* (*Euphorbiaceae*) (Mooibroek and Cornish 2000; van Beilen and Poirier 2007), only a small number of plant species such as *E. ulmoides, Palaquium gutta* (*Sapotaceae*), *Mimusops balata* (*Sapotaceae*), *Achras zapota* (*Sapotaceae*), *Garrya flavescens* (*Garryaceae*), and *G. wrightii* (*Garryaceae*) are known to produce *trans*-polyisoprene rubbers (Schlesinger and Leeper 1951; Roth et al. 1985).

Chemically synthesized *trans*-polyisoprene rubbers are currently utilized as a raw material of golf balls, dental supplies, and submarine cables (Kent and Swinney 1966; Hu 1979). To date, *trans*-polyisoprene rubbers are produced mainly from fossil oil resources, but utilization of biologically synthesized *trans*-polyisoprene rubbers is gaining traction due to recent concerns about global warming and anticipated exhaustion of global oil resources. In view of the ever increasing interest in elucidating the mechanisms that govern the biosynthesis of *trans*-polyisoprene rubbers, the identification of genes involved in *trans*-polyisoprene rubber biosynthesis pathway is an essential step towards understanding and manipulating *trans*-polyisoprene rubber production.

High-molecular-weight (MW) *trans*-polyisoprenes are thought to be synthesized by successive condensation of five-carbon isopentenyl diphosphate (IPP) from low-molecular-weight *trans*-polyisoprenes by a specific prenyltransferase (Fig. 1). Low-MW *trans*-polyisoprenes are biologically synthesized by condensation of IPP with its isomer, dimethylallyl diphosphate (DMAPP). IPP and DMAPP biosynthesis occurs via two different pathways: the mevalonate pathway and the methylerythritol phosphate (MEP) pathway, respectively (Rohmer 1999; Kuzuyama 2002) (Fig. 1). The mevalonate pathway is a 6-step condensation of three acetyl-CoA molecules to IPP via mevalonate (Kuzuyama 2002; Sando et al. 2008a). It occurs in animals, fungi (Goldstein and Brown 1990) and in plant cytoplasm (Newman and Chappell 1999). The MEP pathway utilizes pyruvate and glyceraldehyde 3-phosphate as initial molecules. It consists of seven reaction steps (Sando et al. 2008b) (Fig. 1) and occurs in protozoa, most eubacteria, and plant plastids (Jomaa et al. 1999; Phillips et al. 2008). In plants, cytoplasmic sterols, including many sesquiterpenes and prenyl chains of ubiquinones are formed via the mevalonate pathway in the cytoplasm, whereas isoprenoids such as carotene and lutein are synthesized via the MEP pathway in plastids (Schwender et al. 1996; Lichtenthaler et al. 1997; Disch et al. 1998). Although strong compartmental separation between mevalonate and MEP pathways products in plant cells has long been suspected, evidence of metabolic crosstalk and interaction between the two pathways has started to emerge (Bick and Lange 2003; Hemmerlin et al. 2003; Bamba et al. 2010). Rubbers are synthesized in the cytoplasm, utilizing IPPs produced from both mevalonate and MEP pathways (Bamba et al. 2010). Nevertheless, details of the metabolic pathways for rubber biosynthesis have not yet been completely elucidated; thus some paucity of information on the relevant genes, their regulation and expression persists.

In order to reveal the synthetic mechanisms of *trans*-polyisoprene rubbers in *E. ulmoides*, we have constructed and analyzed expressed sequence tag (EST) libraries and identified several candidates for *trans*-polyisoprene rubber

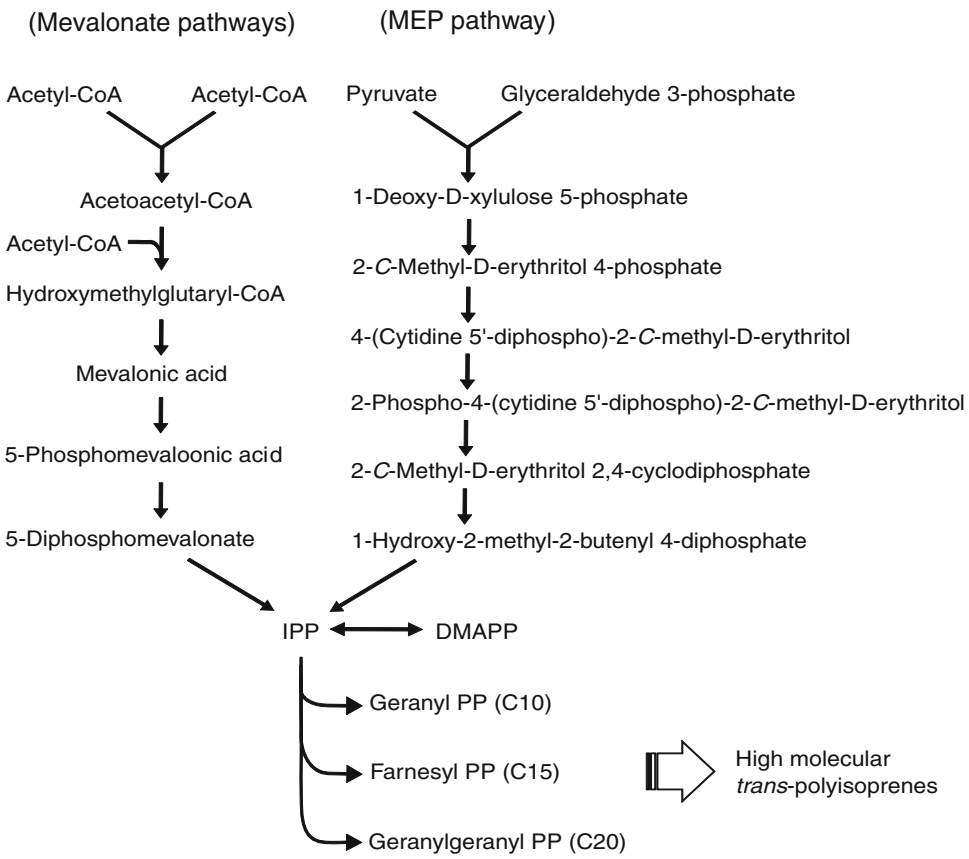

Fig. 1 Putative biosynthetic pathways of *trans*-polyisoprene molecules. IPP and its isomer DMAPP are the starting materials for synthesis of all isoprenoids chemicals including *trans*-polyisoprene. They are synthesized via either the mevalonate or MEP pathways

synthesis. ESTs provide a powerful tool to discover novel genes and facilitate global gene expression profiling in plants, especially in non-model plant species for which full genome sequences are not available (Nieuwenhuizen et al. 2000; Sathiyamoorthy et al. 2010). The genes involved in *trans*-polyisoprene rubber synthesis in *E. ulmoides* are discussed in this study.

Materials and methods

Plant material

To construct cDNA libraries, young current-year stems of *E. ulmoides* Oliv. were collected from a field-grown adult female tree in May in Imabari, Japan (Fig. 2a). The collected stems were immediately separated into two parts, outer stem tissue (OT) and inner stem tissue (IT) (Fig. 2b), frozen in liquid nitrogen at the field, and stored at −80 °C until RNA extraction. OT contained phloem, epidermis, cortex, and laticifers, while IT contained xylem and pith. After sampling, the tree was transplanted in the farm of Kyushu University in Fukuoka, Japan, and conserved as the specimen of genotype. For qPCR and reverse transcription-PCR, *E. ulmoides* tissue samples were harvested from field-grown juvenile trees in November at the Hitachi Zosen Experimental Station (Onomichi, Japan), frozen in liquid nitrogen at the field, and stored at −80 °C until RNA extraction.

RNA extraction

Total RNAs for EST libraries were extracted according to the method described by Chang et al. (1993) with some modifications. The frozen *E. ulmoides* tissues were ground in a mortar and pestle containing liquid nitrogen to a fine powder and immediately transferred into ten volume of extraction buffer [2 % hexadecyltrimethylammonium bromide (CTAB) (Wako, Osaka, Japan), 0.1 M Tris–HCl (pH 9.5) (Wako), 1.4 M NaCl (Wako), 20 mM ethylenediaminetetraacetic acid (EDTA, Wako), 1 % 2-mercaptoethanol (Wako)]. After the solution was incubated for 10 min at 65 °C, an equal volume of chloroform/isoamyl alcohol (24:1) was added and vortexed for 1 min. The solution was subjected to centrifugation and the aqueous phase was transferred into a new tube, and treated with an equal volume of chloroform/isoamyl alcohol again. 1/4 volume of 10 M LiCl (Wako) was added to the aqueous phase and incubated at −20 °C for 2 h. Extract RNA was pelleted by centrifugation for 10 min at 4 °C, dissolved in 450 µl of tris(hydroxymethyl)aminomethane (Wako) (Tris)-EDTA buffer, and treated with an equal volume of

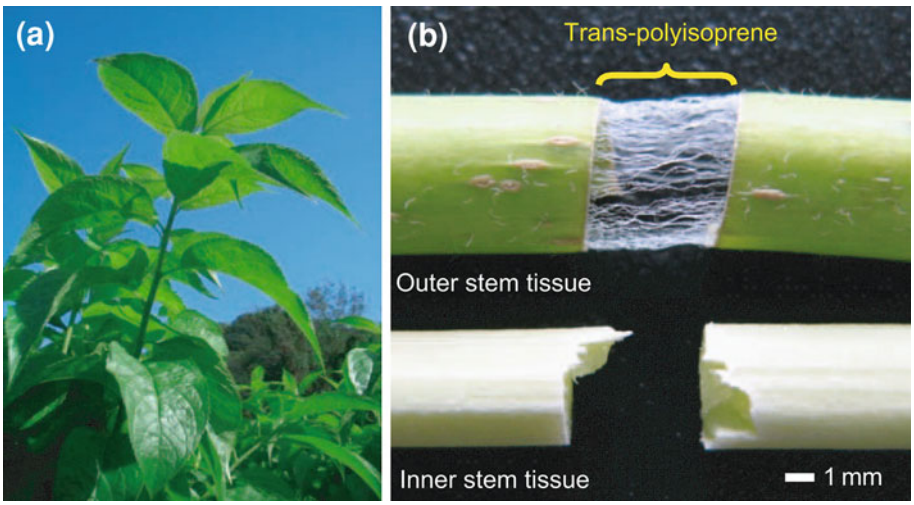

Fig. 2 *E. ulmoides* used in this study. **a** Current year stem and leaves of *E. ulmoides*. **b** To generate EST libraries, stem tissues were separated into outer and inner parts. *Trans*-polyisoprene molecules are typically contained in the outer part of the stem

phenol/chloroform/isoamyl alcohol (25:24:1). RNA was precipitated again by addition of 1/4 volume of 10 M LiCl, pelleted by centrifugation, and resuspended in diethylpyrocarbonate (Sigma-Aldrich, MO) (DEPC)-treated water.

cDNA library construction and EST sequencing

OT and IT cDNA libraries were constructed from respective total RNAs with pGCAP1 cloning vector (Accession, AB191257) according to the vector-capping method (Kato et al. 2005) by Hitachi High-Tech Fielding Corporation (Tokyo, Japan). The inserted cDNAs were sequenced from 5′ ends using M13 (−21) primer (5′-TGTAAAACGACGG CCAGT-3′), DYEnamic ET terminator sequencing kit (GE Healthcare, Buckinghamshire, UK), and MegaBASE 1,000/4,000 sequencers (GE Healthcare). Base-calling was performed using phred program with the quality level set to 20 (Ewing and Green 1998; Ewing et al. 1998). Raw EST data were subjected to trimming of vector sequences using the cross-match program (http://www.phrap.org/). Additional trimmings were conducted where necessary to remove remaining vector sequences. The processed EST sequences were assembled into unigenes using CAP3 program with the high stringency parameters of 40 bases of overlap length and 95 % of overlap identity (Huang and Madan 1999). The assembled EST data were deposited in the GenBank/EMBL/DDBJ (accession FY896671-FY925126).

Functional annotation and classification of unigenes

All similarity searches were executed using BLASTX program with an *E* value cutoff of 10^{-10} (Altschul et al. 1997) against NCBI (http://www.ncbi.nlm.nih.gov/) and The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/index.jsp). Preceding the databank searches, sequences <200 bases were eliminated from query sets and bases with phred quality values <30 were converted to '*N*' characters. Functional classification of the unigenes was performed using Arabidopsis Genome Initiative (AGI) locus codes and the top matches in the TAIR database using the GO slim mapping tool (Berardini et al. 2004) were employed. Putative transcriptional regulators predicted by the AGI locus codes were further annotated using PlnTFDB, an integrative plant transcription factor database (Riaño-Pachón et al. 2007).

3′-Rapid amplification of cDNA ends (RACE)

3′-RACE was performed using 3′-Full RACE Core Set and TaKaRa Ex Taq (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Total RNA extracted from *E. ulmoides* leaves was used as template. Gene-specific primers for first and second PCR were designated using the unigene sequences (Table S1). After first PCR, the reaction mixtures were used as template for second PCR and the products were cloned into the pT7 Blue vector (Merck, Darmstadt, Germany). The sequenced data of second PCR products were assembled with their corresponding unigene sequences.

Yeast complementation experiments

Saccharomyces cerevisiae knock-out strains were purchased and used for functional complementation experiments (Open Biosystems, Huntsville, AL). Growth and manipulation of *S. cerevisiae* were performed as described previously (Rose et al. 1990). The DNA fragments encoding complete ORF sequences were obtained by PCR and inserted into the pYES2 expression vector (Invitrogen, Carlsbad, CA). Transformation, sporulation of transformants, and complementation assay using haploid cells were

performed as described previously (Sando et al. 2008a, b). The yeast strains and primers used in this study are listed in Table S2 and S3.

Quantitative real-time PCR (qPCR)

For qPCR experiments, total RNAs were extracted from various *E. ulmoides* tissues (lateral buds, shoots) using RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with some modifications. Frozen tissues (0.10–0.15 g) were ground in liquid nitrogen to a fine powder and transferred into 450 µl buffer RLC supplemented with 5 % β-mercaptoethanol and 2.5 % polyvinylpyrrolidone (PVP) 40. An equal volume of chloroform was added to the lysate and vortexed. The mixture was centrifuged, and separated aqueous phase was transferred to a new tube and used for the subsequent RNA extraction step according to RNeasy Plant Mini kit protocol. On-column DNase digestion step described in the RNeasy Mini Handbook (Qiagen) was further included using RNase-free DNase set (Qiagen). Extracted total RNAs were quantified by Spectrophotometer DU640 (Beckman, CA, USA) and stored at −80 °C until use.

cDNA synthesis was carried out using PrimeScript RT reagent kit (Takara Bio) and qPCR experiments were performed using the Mx3000P real-time PCR system with 25 µl of reaction volume (Agilent Technologies, Santa Clara CA, USA) and SYBR *Premix Ex Taq* (Takara Bio), according to the manufacturer's instructions. Primers were designed using the Primer Express software (Applied Biosystems) (Table S4). The target gene transcripts were normalized to the reference gene transcripts (Glyceraldehyde-3-phosphate dehydrogenase gene, EST number RB033_K16.b) in the same RNA samples.

Results

Construction of cDNA library and EST annotation

Since previous studies have demonstrated that high-molecular-weight *trans*-polyisoprene rubber in *E. ulmoides* is accumulated in the outer part of stems (Bamba et al. 2002), two cDNA libraries derived from an outer and inner stem tissue (OT, IT) of a young current-year stem were constructed. Of these, 17,914 OT and 20,253 IT clones were randomly picked from their respective libraries and sequenced using the Sanger method. After removing vector sequence, polyA tail, and low-quality sequences, a total of 27,752 high-quality sequences were retained (Table 1). Clustering and assembly of the sequenced data were subsequently carried out and 10,520 unigenes consisting of 4,302 contigs and 6,218 singletons were obtained (Table 1). The average length of contig was 751.2 bp, which was longer than the average length of singleton (580.6 bp). The lengths of contigs ranged from 202 to 2311 bp, and those of singletons from 193 to 953 bp, respectively.

The *E. ulmoides* unigenes were annotated using the BLASTX program against NCBI nr and TAIR database, revealing 7,869 (74.8 %) NCBI and 7,743 (73.6 %) TAIR matches with an *E* value 10^{-10} or better. Among these, 872 genes exclusively expressed in the OT and 782 exclusively expressed in the IT were identified. Genes with roles in other cellular components, response to stress, and response to abiotic or biotic stimulus were frequent in the OT library, whereas genes of mitochondrial origin were prevalent in the IT library (Fig. S1). Moreover, no genes of plastid or extracellular were found in the IT library (Fig. S1). Two OT and five IT genes encoding homologues of APL and NST1, transcriptional factors which function as sieve-tubes or in xylem development (Table 2), were identified.

A homologue each of major latex protein (MLP)-encoding and metallothionein-encoding genes shared large parts of both OT and IT libraries, indicating strong expression of both proteins in the stem tissue (Table 3). Homologues of 6 kinds of rubber particle membrane proteins (RPMPs) which are important for effective synthesis of high-molecular poly-isoprene in latex (Dennis and Light 1989; Goyvaerts et al. 1991) were also detected in the both libraries, indicating the necessity of these proteins for high-molecular-weight *trans*-polyisoprene synthesis in *E. ulmoides* (Table 4). A phylogenetic analysis of known RPMPs divided *E. ulmoides* RPMPs roughly into two groups, suggesting the presence of different types of RPMPs in *E. ulmoides* (Fig. 3a).

Trans-polyisoprene rubbers in *E. ulmoides* are made by head-to-tail condensation exclusively in *trans* configuration of IPP (Bamba et al. 2010). The details of the IPP synthesis pathways in plant have been described elsewhere

Table 1 Summary of constructed *E. ulmoides* EST libraries

Category	No. of sequenced DNAs
Total ESTs	38,167
Outer part library	17,914
Inner part library	20,253
High quality ESTs	27,752
Unigenes	10,520
Contigs	4,302
Singletons	6,218

Outer part and inner part cDNA libraries derived from outer and inner stem tissue (OT, IT) were constructed and sequenced

Table 2 Homologous genes of transcriptional factors involved in sieve-tube or xylem development in OT and IT libraries

Unigene ID	No. of ESTs in each library		Similar proteins	Gene families	Function	*E* value[a]	Reference
	OT	IT					
Contig1288	6	0	ZeHB3	HB	Phloem formation	1.00E−24	Nishitani et al. 2002
Contig295	2	0	APL	G2-like	Phloem formation/suppression of xylem formation	9.00E−61	Bonke et al. 2003
RT060_J18	0	1	CNA/ATHB15	HB	Suppression of xylem formation	1.00E−67	Ohashi-Ito and Fukuda 2003
RT015_F10	0	1	REV/IFL1	HB	Xylem formation	1.00E−116	Zhong and Ye 1999
RT026_A22	0	1	REV/IFL1	HB	Xylem formation	3.00E−49	Zhong and Ye 1999
Contig3125	0	2	NST1	NAC	Xylem fiber formation	2.00E−75	Zhong et al. 2007
Contig366	0	5	NST1	NAC	Xylem fiber formation	4.00E−87	Zhong et al. 2007

BLASTX search was carried out against TAIR protein database

[a] *E* value of the best hit

Table 3 Homologues of genes encoding major latex protein (MLP) and metallothionein (MT)

Unigene ID	No. of ESTs in each library			Similar protein	*E* value[a]
	OT	IT	Total		
Contig251	145	215	360	Metallothionein type 2	1.00E−36
Contig274	223	85	308	Major latex protein (MLP)	1.00E−39
Contig1621	105	39	144	Dehydrin	1.00E−26
Contig2531	26	67	93	Glutamate-rich protein	3.00E−17
Contig271	67	16	83	γ-Thionin	1.00E−26
Contig1726	47	30	77	Auxin-repressed/dormancy-associated protein	6.00E−47
Contig2894	11	52	63	Arabinogalactan-protein	1.00E−67
Contig347	25	29	54	Glycine-rich RNA-binding protein	3.00E−65
Contig192	34	17	51	Cold-induced plasma membrane protein	2.00E−22

[a] *E* value of the best hit

(Kuzuyama 2002). As far as IPP biosynthesis is concerned, an IPP isomerase gene, the DXP (deoxy-xylulose phosphate) synthase-encoding gene of the MEP pathway, and all genes of the mevalonate pathway except that encoding phosphomevalonate kinase were present in the OT and IT libraries (Table 5). It is noteworthy to mention that genes encoding five isoforms of farnesyl diphosphate (FPP) synthases were identified in the libraries. Phylogenetic analysis could place two among them on one branch that comprises also AtFPS1 and AtFPS2 in *Arabidopsis*, but the other three on a different branch (Fig. 3b). Although the last steps of *trans*-polyisoprene rubber synthesis have not yet been identified, the rubber is thought to be produced by mechanisms similar to those of short-chain *trans*-polyprenyl diphosphates such as FPP and geranylgeranyl diphosphate (GGPP). Genes encoding the enzymes described above were designated as *EuACAT1, 2, EuHMGS1, 2, EuHMGR1, 2, EuMVK1, EuMVD1, EuIDI1, EuDXS1-4, and EuTIDS1-9* (*E. ulmoides trans*-isoprenyl diphosphate synthase), respectively (Table 5).

Functional analysis of isoprenoids synthesis genes

Since many EST sequences associated with *trans*-polyisoprene rubber biosynthesis were obtained, primers for 3′-RACE were designed and full-length cDNAs of acetyl-CoA acetyltransferase (*EuACAT1, 2*), 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (*EuHMGS1, 2*),

HMG-CoA reductase (*EuHMGR1, 2*), mevalonate kinase (*EuMVK1*), diphosphomevalonate decarboxylase (*EuMVD1*), IPP isomerase (*EuIDI1*), FPP synthase (*EuTIDS2, 4*), and FPP synthase-like (*EuTIDS1, 3, 5*) were isolated (Table 5).

Sequence analysis indicated that IPP isomerase was highly identical to other plant IPP isomerases, particularly those of *Ipomoea* sp. (92 %), *Nicotiana tabacum* (93 %), and *Solanum lycopersicum* (93 %). As for acetyl-CoA acetyltransferase, the two cDNAs cloned encoded enzymes which were 89 % identical to each other. Both showed high similarities to acetyl-CoA acetyltransferase of *Camellia oleifera* (90 %), *Bacopa monnieri* (87 %), and *H. brasiliensis* (86 %). Two cDNAs encoding HMG-CoA synthase were 86 % identical to each other and more than 84 % identical to those of *Camptotheca acuminate*, *Ricinus communis*, and *H. brasiliensis* HMG-CoA synthases. Two HMG-CoA reductases, one diphosphomevalonate decarboxylase and one mevalonate kinase cDNA were obtained and the diphosphomevalonate decarboxylase showed 86, 87, and 84 % identity to *R. communis*, *Populus trichocarpa*, *H. brasiliensis*, respectively. However, HMG-CoA reductases and mevalonate kinase were <80 % identical to known HMG-CoA reductases and mevalonate kinases. The C-terminal regions of HMG-CoA reductases contained more crucial motifs to activity and were highly conserved in the *E. ulmoides* HMG-CoA reductase. All five FPP synthases contained the aspartate-rich motif consisting of DDXXD which is conserved in *trans*-polyprenyl diphosphate synthases (Wang and Ohnuma 1999), and the two (*EuTIDS2, 4*) which constitute the AtFPS1 and 2 branch showed 85 % more identity to plant FPP synthases of *H. brasiliensis,* and *Panax ginseng*. The other three FPP synthases (*EuTIDS1, 3, 5*) were <80 % identical to known plant FPP synthases. As a distinctive feature, the 3 FPP synthases shared an additional six amino acid insertion approximately 60 residues downstream of N terminus (Fig. 4).

To analyze the functions of these genes, the cDNAs were ligated into pYES2 and placed under the GAL1 promoter. The resultant plasmids were integrated into *S. cerevisiae* mutants and transformants were inoculated on solid YPD medium plates. Resulting transformants harboring the respective genes, *EuACAT1, 2, EuHMGS1, EuMVK1, EuMVD1, EuIDI1,* and *EuTIDS2, 4* were able to suppress the growth deficiency phenotype of the corresponding mutants (Fig. 5). However, *EuHMGS2 and EuTIDS1, 3, 5* could not (data not shown), indicating that these, in contrast to the other eight, could not functionally complement corresponding disrupted genes in *S. cerevisiae*. The genes *EuHMGR1, 2, EuDXS1–4, and EuTIDS6–9* were

Table 4 Homologous genes of rubber particle membrane proteins in OT and IT libraries

Unigene ID	No. of ESTs in each library			(SRPP) *E* value[a]	(REF) *E* value[a]
	OT	IT	Total		
Contig196	5	1	6	3E−05	2E−09
Contig1073	14	17	31	3E−18	7E−24
Contig2579	0	5	5	1E−18	1E−26
Contig2846	6	4	10	3E−03	1E−04
Contig4112	0	2	2	1E−03	3E−06
RB047_K12	1	0	1	4E−05	9E−07

The *E* values were calculated against *H. brasiliensis* SRPP (AAO66432) and REF (AAP46159), respectively

[a] *E* value of the best hit

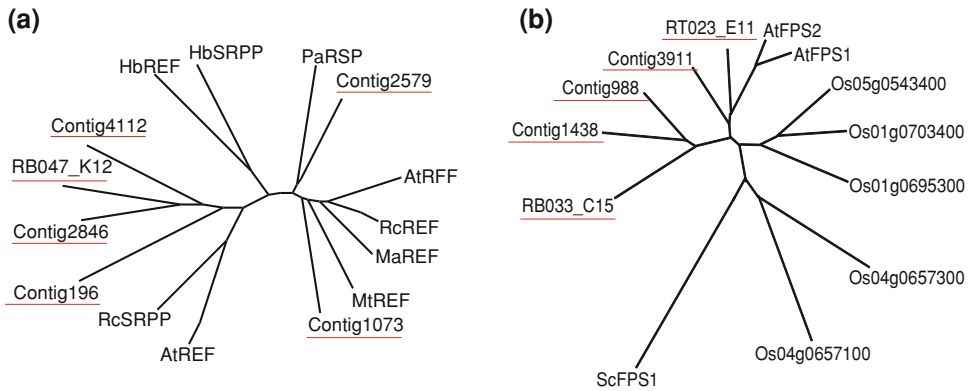

Fig. 3 Phylogenetic trees of **a** RPMP and **b** FPP synthases. Sequences derived from *E. ulmoides* are underlined (in *red*). Analysis was performed using Clustal X 2.0 (Larkin et al. 2007). The accession numbers of the sequences are as follows: **a** *H. brasiliensis* SRPP (AY237009), REF (AY430052), *Parthenium argentatum* RSP (AF541942), *Ricinus communis* SRPP (XM_002514871), REF (XM_002512381), *A. thaliana* RFF (NM_111423), REF (NM105404), *Morus alba* REF (GQ466080), *Medicago truncatula* REF (AC149135), **b** *A. thaliana* FPS1 (AAF44787), FPS2 (AAB07247), *Oryza sativa* 01g0695300 (NP_001043959), 01g0703400 (NP_001044000), 04g0657100 (NP_001054121), 04g0657300 (NP_001054122), 05g0543400 (NP_001056204)

Table 5 Identified putative genes of isoprenoid-biosynthesis in *E. ulmoides*

Unigene ID	No. of ESTs in each library			Designated gene name	Actual or putative product	Top match	
	OT	IT	Total			Accession number[a]	*E* value[b]
MVA (mevalonate) pathway							
RB047_D18	1	0	1	*EuACAT1*	Acetyl-CoA acetyltransferase	BAF98277	1E−67
RT050_P19	0	1	1	*EuACAT2*	Acetyl-CoA acetyltransferase	BAF98277	5E−68
Contig1491	1	3	4	*EuHMGS1*	HMG-CoA synthase	CAO63650	5E−118
Contig3771	1	1	2	*EuHMGS2*	HMG-CoA synthase	ABV02025	5E−103
Contig2465	0	3	3	*EuHMGR1*	HMG-CoA reductase	AAB87727	2E−55
RT032_A10	0	1	1	*EuHMGR2*	HMG-CoA reductase	P48021	2E−49
Contig298	4	0	4	*EuMVK1*	Mevalonate kinase	CAO17636	1E−78
Contig3459	1	3	4	*EuMVD1*	Diphosphomevalonate decarboxylase	CAO64147	2E−72
Contig861	1	2	3	*EuIDI1*	IPP isomerase	O48965	7E−96
MEP (methylerythritol phosphate) pathway							
Contig3476	1	1	2	*EuDXS1*	DXP synthase	CAO44179	1E−38
RB015_F22	1	0	1	*EuDXS2*	DXP synthase	O78328	1E−60
RB058_N12	1	0	1	*EuDXS3*	DXP synthase	O78328	4E−130
RT016_L11	0	1	1	*EuDXS4*	DXP synthase	BAF98289	3E−60
Trans-isoprenyl diphosphate synthase							
RT061_D23	0	1	1	*EuTIDS6*	GPP synthase	ACC77966	7E−47
Contig2314	1	1	2	*EuTIDS7*	GPP synthase (small subunit)	AAS82859	2E−47
RT020_C19	0	1	1	*EuTIDS8*	GPP synthase (large subunit)/GGPP synthase	Q42698	7E−14
RT016_K07	0	1	1	*EuTIDS9*	GGPP synthase-related protein	CAO64763	2E−62
Contig3911	3	2	5	*EuTIDS2*	FPP synthase	BAB60822	2E−127
RT023_E11	0	1	1	*EuTIDS4*	FPP synthase	AAK63847	3E−127
Contig988	3	0	3	*EuTIDS1*	FPP synthase-like	BAB16687	3E−91
Contig1438	1	1	2	*EuTIDS5*	FPP synthase-like	BAB16687	2E−118
RB033_C15	1	0	1	*EuTIDS3*	FPP synthase-like	AAN62522	2E−90

BASTX search was carried out against NCBI protein database

[a] Accession number of the best hit

[b] *E* value of the best hit

not tested because appropriate mutants could not be obtained.

Expression of *EuTIDS* genes in *E. ulmoides*

The expression of five *EuTIDS* genes was measured by qPCR in young and mature stems, buds, and immature and mature leaves. *EuTIDS2* and *4,* which complemented FPP synthases deficiency in yeast, were mainly expressed in the mature leaf, mature bud, and immature leaf (Fig. 6). *EuTIDS4* expressed at high levels in all tissues compared with *EuTIDS2* (Fig. 6). Although the functions of *EuTIDS1, 3,* and *5* are not known, *EuTIDS1* was highly expressed in the mature leaf, whereas *EuTIDS5* was strongly expressed in the mature bud and immature leaf (Fig. 6). Similarly, expression of *EuTIDS3* was detected both in the bud and the immature leaf, but the expression level was low compared with that of *EuTIDS5* (Fig. 6). Overall, expression patterns of the five *EuTIDSs* in these tissues were individually distinct, suggesting that *EuTIDSs* having different roles in *E. ulmoides*.

Discussion

We generated and analyzed *E. ulmoides* EST libraries, and one of the characteristic results obtained was the high expression of MLP homologues and metallothionein (Table 3). MLP was first identified in the latex of opium poppy (*Papaver somniferum*) (Nessler and Burnett 1992) and later found in several plants such as peach and strawberry. The functions of MLPs are not known yet, but MLPs are considered to form a superfamily with intracellular pathogenesis-related (IPR) proteins due to their structural similarities (Lytle et al. 2009). Since more abundant clones were obtained in the OT library than in the

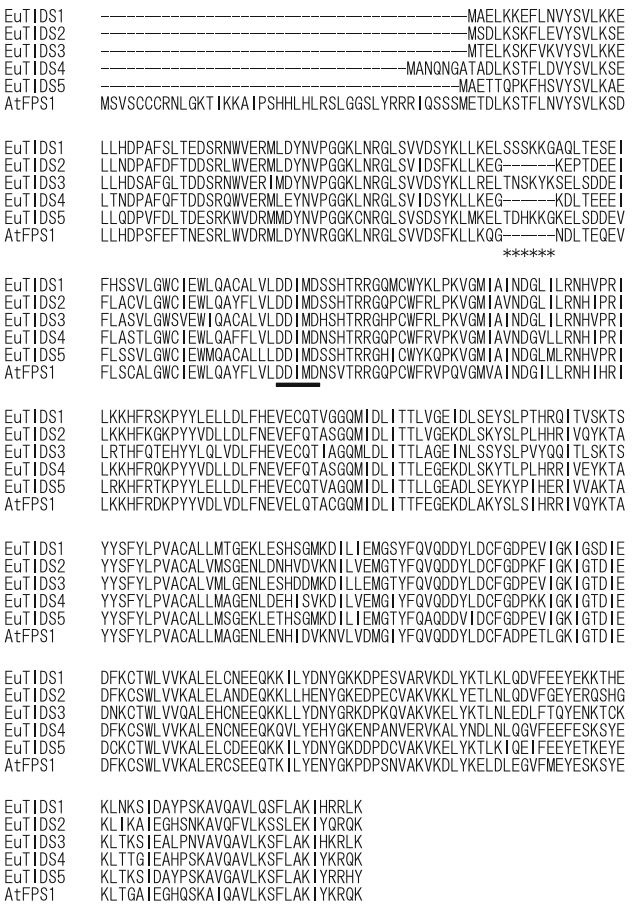

Fig. 4 Amino acid alignment of EuTIDS1–5 against AtFPS1. Stars denote the 6 additional amino acid insertions of TIDS1, 3 and 5. *Bold underbar* denotes the conserved DDXXD motif in *trans*-polyprenyl diphosphate synthases

IT library (Table 3) and latex, which is considered to have a protective role in plants and that accumulated in the outer tissues of *E. ulmoides,* MLPs might have some protective role in *E. ulmoides*. Another strongly expressed gene, metallothionein is generally considered to be deeply involved with copper tolerance and homeostasis, and is expressed at very high levels in many plant species (Cobbett and Goldsbrough 2002). Although the exact role of metallothionein in plant tissues remains to be verified, metallothionein should be working and expressed at high levels for homeostasis in *E. ulmoides* just like in other plants.

Another characteristic point of *E. ulmoides* ESTs was that six kinds of genes encoding RPMPs including REF (rubber elongation factor) and SRPP (small rubber particle protein) were identified (Table 4). Six homologous genes encoding RPMPs are considered necessary for effective synthesis of *cis*-polyisoprene rubbers in *H. brasiliensis* and in *Parthenium argentatum* (Dennis and Light 1989; Kim et al. 2004), and were identified at a high rate in *H. brasiliensis* EST library (Ko et al. 2003). Although the *E. ulmoides* rubber is composed of *trans*-polyisoprenes, *cis*- and *trans*-prenyltransferases catalyze similar reactions of sequential condensation of isopentenyl diphosphate (IPP) (Koyama 1999). Therefore, the RPMPs should function to enhance *trans*-polyisoprene synthesis in *E. ulmoides* in the same manner as they do in *cis*-polyisoprene synthesis in *H brasiliensis*.

To date, high-molecular-weight *trans*-polyisoprene rubber synthase has not been identified. Genes encoding five kinds of putative FPP synthases were obtained in the EST libraries and two (EuTIDS2 and 4) of them that could complement FPP deficient yeast indicated that they functioned as FPP synthases (Figs. 4, 5). However, the other three putative FPP synthases, EuTIDS1, 3, and 5 encoded seven conserved domains of *trans*-polyprenyl diphosphate synthases including the aspartate-rich motif, but could not complement FPP synthase-deficient yeast (Fig. 5). Various *trans*-polyprenyl diphosphate synthases including farnesyl diphosphate (C15), geranylgeranyl diphosphate (C20), presqualene diphosphate (C30), octaprenyl pyrophosphate (C40), and undecaprenyl pyrophosphate (C55) synthase, have already been identified and characterized (Fujisaki et al. 1986; Nakashima et al. 1995; Cunillera et al. 1996; Okada et al. 2000). In *trans*-polyprenyl diphosphate synthase, the aspartate-rich motif, DDXXD, is commonly found, and mutagenic studies and three-dimensional structure analysis have indicated that the isoprene chain length is controlled by the direct interaction of fourth or fifth amino acid residues upstream of the aspartate-rich motif (Tarshis et al. 1996; Wang and Ohnuma 1999). The aromatic residues upstream of the aspartate-rich motif in FPP synthase limit the product length to C15 (Ohnuma et al. 1997), but a lack of these bulky amino acid residues elongates the product chain length to a range between C30 and C50 (Ohnuma et al. 1998; Okada et al. 1998; Hirooka et al. 2000). EuTIDS2 and 4 encoded phenylalanine residues as the fourth or fifth amino acids, respectively, before the aspartate-rich motif; however, the other three, EuTIDS1, 3, and 5 encoded cysteine and alanine residues at this positions (Fig. 4). Therefore, these enzymes did not function as FPP synthases, but might be functional as long chain *trans*-polyprenyl diphosphate synthases in *E. ulmoides*. Since DNA sequences of long-chain *trans*-polyprenyl diphosphate synthases and their functional mechanisms are not known yet, EuTIDS1, 3, and 5 should be good candidates to study *trans*-polyisoprene rubber synthase in *E. ulmoides*. Their expression levels in stem, bud, and leaf (Fig. 6) also suggest their involvement in *trans*-polyisoprene rubber synthesis due to the prevalent accumulation of *trans*-polyisoprene rubber in these tissues. Further studies of these genes are required.

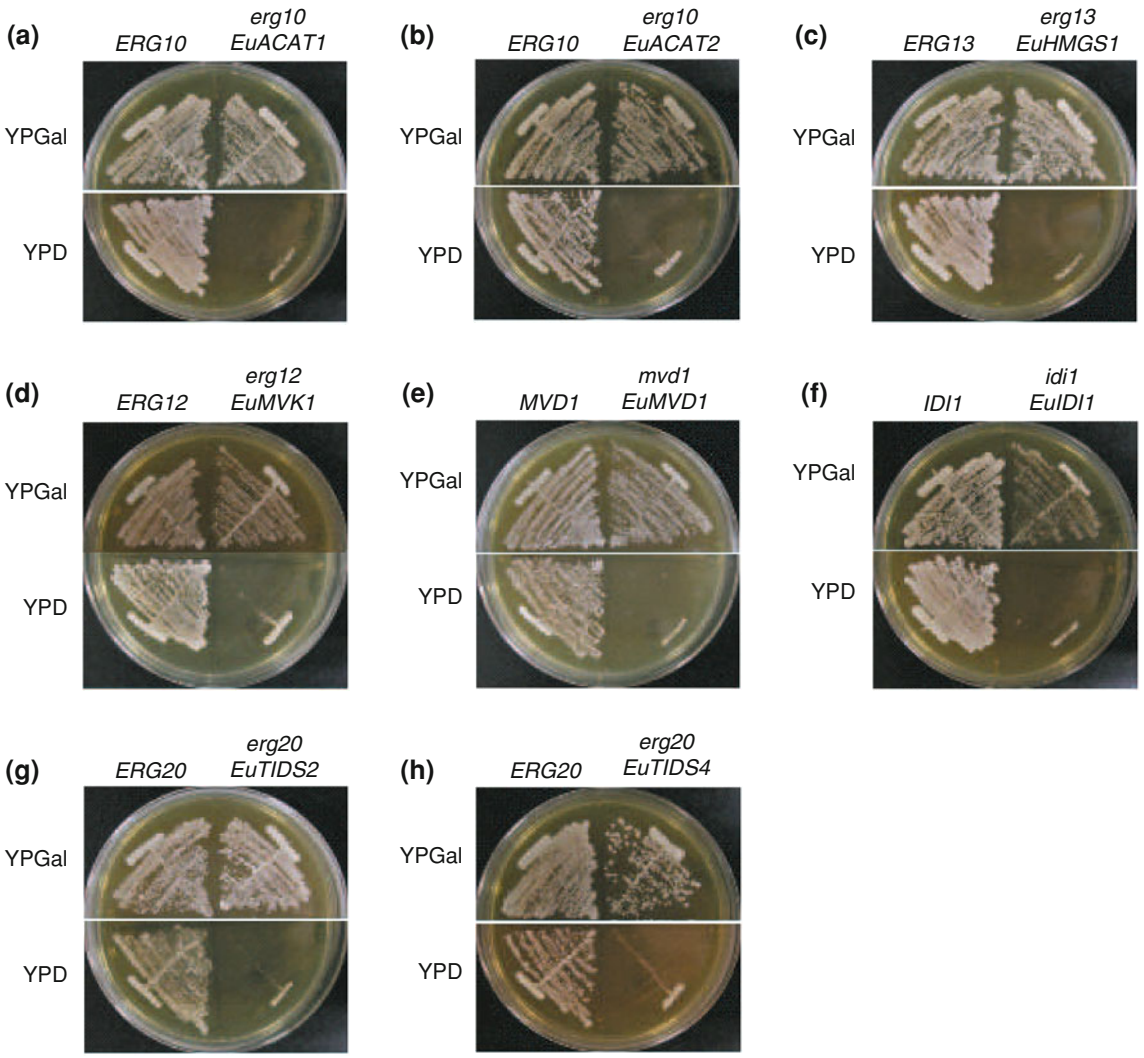

Fig. 5 Functional complementation analysis of *E. ulmoides* genes. Yeast, *erg10*, *erg12*, *erg13*, *erg20*, *mvd1* and *idi1* disrupted strains were transformed with EuACAT1, EuACAT2, EuMVK1, EuHMGS1, EuTIDS2, EuTIDS4, EuMVD1 and EuIDI1 expression vector, respectively (**a**–**h**). Haploid wild-type (*left*) and their disruptant strains transformed by corresponding vectors (*right*) were streaked on solid YPGal and YPD medium. The *erg13* and *erg20* disrupted strains were also transformed with EuHMGS2 and EuTIDS1, 3, 5, respectively, but no complementation was observed (data not shown)

IPP molecules are a basic unit for *trans*-polyisoprene rubbers and *trans*-polyprenyl diphosphate synthesis, and the study of the genes related to IPP synthesis is also an important target to investigate *trans*-polyisoprene rubber synthesis. Previous work indicates that high-molecular-weight *cis*-polyisoprene is synthesized using IPP molecules synthesized via the mevalonate pathway, not via the MEP pathway in *H. brasiliensis* (Sando et al. 2008b). However, both MEP and mevalonate pathways could supply IPP in the biosynthesis of *trans*-polyisoprene in *E. ulmoides* (Bamba et al. 2010). In our EST libraries, all mevalonate pathway genes except that encoding phosphomevalonate kinase could be isolated, but only a DXP synthase-encoding gene of the MEP pathway was obtained. Since MEP pathway is confined to plastids in the plant cell and the EST libraries were constructed using the stem tissues, abundance of mRNA molecules of MEP pathway genes was low and their cloning difficult, suggesting that the mevalonate pathway dominates IPP synthesis in *E. ulmoides* stem. Therefore, *trans*-polyisoprene rubbers in the stem might be synthesized using IPP molecules derived from mevalonate pathway products.

In addition, almost 8,000 unigenes which could be annotated with functionally known genes were isolated. Our EST data could allow constructing microarrays for large-scale examination of gene expression of *trans*-polyisoprene rubber synthesis. This study should provide a valuable resource for further studies of *trans*-polyisoprene

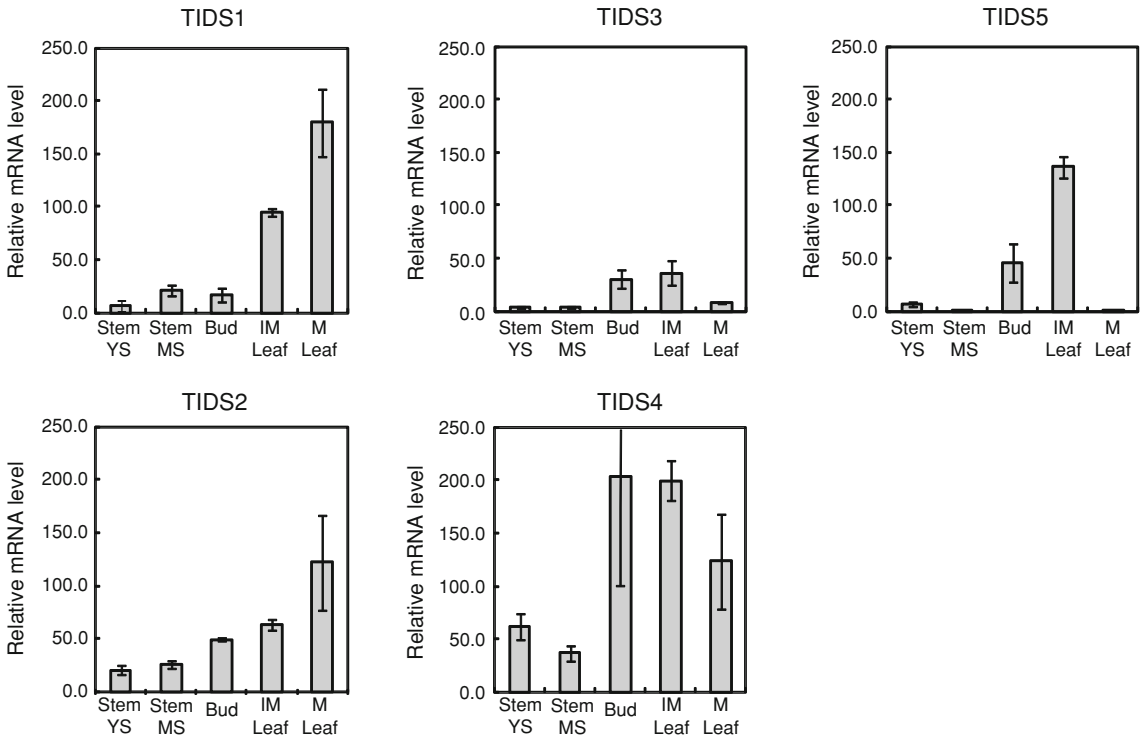

Fig. 6 Relative mRNA levels of TIDS genes were analyzed by qPCR. mRNAs were isolated from field grown young and mature stems, buds, and immature and mature leaves. Purity and amount of extracted RNAs ranged from 2.12 to 2.30 (A_{260}/A_{280}) and from 345 to 627 ng/μl, respectively. A cDNA derived from 100 ng RNA was used for each qPCR reaction. All measurements were carried out using three different plants. C_q values of TIDS1—5 and reference gene were 29.94, 25.92, 27.63, 28.30, 26.92, and 25.58, respectively. Both actin (RT045_H16.b) and GAPDH (RB033_K16.b) gene which are frequently used as a reference of transcripts for qPCR were tested. Since GAPDH mRNA level was more stable than that of actin (data not shown), GAPDH was used as reference gene in *E. ulmoides.* YS, MS, IM leaf, and M leaf indicate young stem, mature stem, immature leaf, and mature leaf, respectively. The mRNA levels are expressed as relative numbers to the reference gene

rubber synthesis in *E. ulmoides*, e. g. a marker gene to analyze *E. ulmoides* strains.

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