Supporting Information

Li-Beisson et al. 10.1073/pnas.0909090106

SI Materials and Methods

T-DNA Insertional Mutant Identification and Isolation. All T-DNA insertional mutant lines (1) for genes of interest were identified using the SIGnAL T-DNA Express *Arabidopsis* Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) provided by the Salk Institute Genomic Analysis Laboratory. Individual seeds for these lines were obtained from the Arabidopsis Biological Resource Center at The Ohio State University. Plants were grown from these seeds, and for each line DNA was prepared from leaves and used for genotype screening. For T-DNA insertion in the *CYP77A6* gene, lines SALK_019080C and SALK_023926C were identified and homozygous plants were obtained by PCR with a combination of gene-specific primers CYP77A6-tF, CYP77A6-tR, and T-DNA left border primer LBa1, respectively. Homozygous plants isolated from SALK_019080C are named *cyp77a6–1* and from SALK_023926C are named *cyp77a6–2*. For T-DNA insertion in the *GPAT6* gene, SALK_136675 and SALK_146013 were identified and homozygous plants were obtained by PCR with a combination of gene-specific primers GPAT6-tF, GPAT6-tR, and T-DNA left border primer LBa1, respectively. Homozygous plants isolated from SALK_136675 are named *gpat6–1*, and the other line from SALK_146013 is named *gpat6–2*. For T-DNA insertion in the *CYP86A4* gene, SALK_073078 and SALK_077857 were identified and homozygous plants were obtained by PCR with a combination of gene-specific primers CYP86A4-tF, CYP86A4-tR, and T-DNA left border primer LBa1, respectively. These mutants in sequential order as above are named *cyp86a4–1* and *cyp86a4–2*. (Primer sequences are presented in Table S1.)

Total RNA Isolation and Gene Transcript Analysis by RT-PCR. Total RNA was extracted from inflorescences of WT, *gpat6–1*, *gpat6–2*, *cyp86a4–1*, *cyp86a4–2*, *cyp77a6–1*, and *cyp77a6–2* plant lines by grinding these tissues in liquid nitrogen followed by RNA extraction with the Plant RNeasy Mini kit from Qiagen. Extracted RNA was quantified by nanodrop. To eliminate any contaminating DNA, RNA preparations were treated with DNase using the DNA-free kit from Ambion, and the treated RNA (1–2 μg) was subjected to reverse transcription using the SuperScript II first-strand synthesis system for RT-PCR (Invitrogen). Gene transcripts for GPAT6, CYP86A4, CYP77A6, GPAT4, and CYP77A7 were amplified using primers GPAT6-rtF and GPAT6-rtR, CYP86A4-F2 and CYP86A4-R1, CYP77A6-rtF and CYP77A6-rtR, GPAT4-F and GPAT4-R, and CYP77A7-rtF and CYP77A7-rtR with amplification of the initiation factor eIF4A-1 (At3g13920) as a control with primers EIF4A-F and EIF4A-R (for primer sequences, see Table S1).

***35S:GPAT6* Overexpression Construct and Plant Transformation.** Genomic DNA was prepared from *Arabidopsis* leaf tissue using a plant mini DNA kit according to the manufacturer's instructions (Qiagen). Genomic DNA sequences encoding the *GPAT6* gene were amplified by PCR using forward primer GPAT6-cF and reverse primer GPAt6-cR (see Table S1 for sequences). The PCR product was cloned as an NcoI-SpeI fragment into binary vector pCAMBIA1302 (CAMBIA). The construct (*35S::GPAT6*) was introduced into *Agrobacterium tumefaciens* strain C58C1 for *Arabidopsis* vacuum infiltration.

CYP77A6 Expression and in Vitro Activity Assay. ***Cloning of CYP77A6.*** The PCR was carried out with Isis DNA polymerase (Qbiogene) and for 30 thermal cycles (1 min at 96 °C, 2 min at 54 °C, 2 min at 72 °C). After addition of adenine nucleotides on each side of the PCR product by an additional step with *Taq* polymerase (10 min at 72 °C), purified PCR product was cloned into PCRII TOPO vector (Invitrogen) and transferred in the pYeDP60 vector using the BamHI and KpnI restriction sites. The sequence was verified by DNA sequencing after the cloning step in the PCRII TOPO vector.

Enzyme activities. Yeast cultures were grown and *CYP77A6* expression was induced as described in Pompon et al. (2) from one isolated transformed colony. After growth, cells were harvested by centrifugation and manually broken with glass beads (0.45 mm diameter) in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 600 mM sorbitol. The homogenate was centrifuged for 10 min at 10,000 $\times$ *g*. The resulting supernatant was centrifuged for 1 h at 100,000 $\times$ *g*. The pellet consisting of microsomal membranes was resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 30% (vol/vol) glycerol with a Potter-Elvehjem homogenizer and stored at −30 °C. The volume of resuspension buffer is proportional to the weight of yeast pellet: Microsomes extracted from 6 g of yeast are resuspended in 3 mL of buffer. All procedures for microsomal preparation were carried out at 0–4 °C.

Thin layer chromatographic (TLC) methods. Incubation media were directly spotted on TLC plates. For separation of metabolites from residual substrate, TLCs were developed with a mixture of diethyl ether/light petroleum (boiling point, 40°–60 °C)/formic acid (50:50:1, vol/vol/vol). The plates were scanned with a radioactivity detector (Raytest RITA Star).

GC-MS analysis. GC-MS analyses were carried out on a gas chromatograph (Agilent 6890 Series) equipped with a 30-m capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 μm (HP-5MS). The gas chromatograph was combined with a quadrupole mass selective detector (Agilent 5973N). Mass spectra were recorded at 70 eV and analyzed as in Eglinton et al. (3).

For analysis of products generated by recombinant CYP77A6 in incubation with 16-hydroxypalmitic acid, incubation media were extracted twice with 0.5 mL of diethyl ether, and metabolites were methylated with diazomethane, trimethylsilylated with *N,O*-bistrimethylsilyltrifluoroacetamide containing 1% (vol/vol) trimethylchlorosilane (1:1, vol/vol), and subjected to GC-MS analysis. The mass spectrum of derivatized metabolites showed ions at *m/z* (percentage of relative intensity) as follows: 73 (100%) $(CH_3)_3Si^+$, 75 (53%) $((CH_3)_2Si^+{=}O)$, 146 (5%) $(CH_2{=}C^+(OSi(CH_3)_3{-}O\text{-}CH_3)$, 159 (10%) $(CH_3{-}O^+{=}C^+(OSi(CH_3)_3)CH{=}CH_2)$, 399 (5%) (*M*-47) [loss of methanol from the (M-15) fragment], 415 (2%) (M-31) (loss of OCH_3 from the methyl ester), and 431 (6%) (M-15) (loss of CH_3 from TMSi group). We also observed ions at *m/z* (percentage of relative intensity) as follows: 261 (3%), 287 (7%) (characteristic of the derivatized 11,16-dihydroxypalmitic acid), 275 (1.5%), 273 (3%) (characteristic of derivatized 10,16-dihydroxypalmitic acid), 289 (13%), 259 (30%) (characteristic of derivatized 9,16-dihydroxy palmitic acid), 303 (38%), and 245 (89%) (characteristic of derivatized 8,16-dihydroxypalmitic acid).

1. Alonso JM, et al. (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301:653–657.
2. Pompon D, Louerat B, Bronine A, Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. *Methods Enzymol* 272:51–64.
3. Eglinton G, Hunneman D, McCormick A (1968) Gas chromatography-mass spectrometry studies of long-chain hydroxy acids III. The mass spectra of the methyl esters TMS ethers of aliphatic hydroxy acids. A facile method of double bond location. *Org Mass Spectrom* 1:593–611.

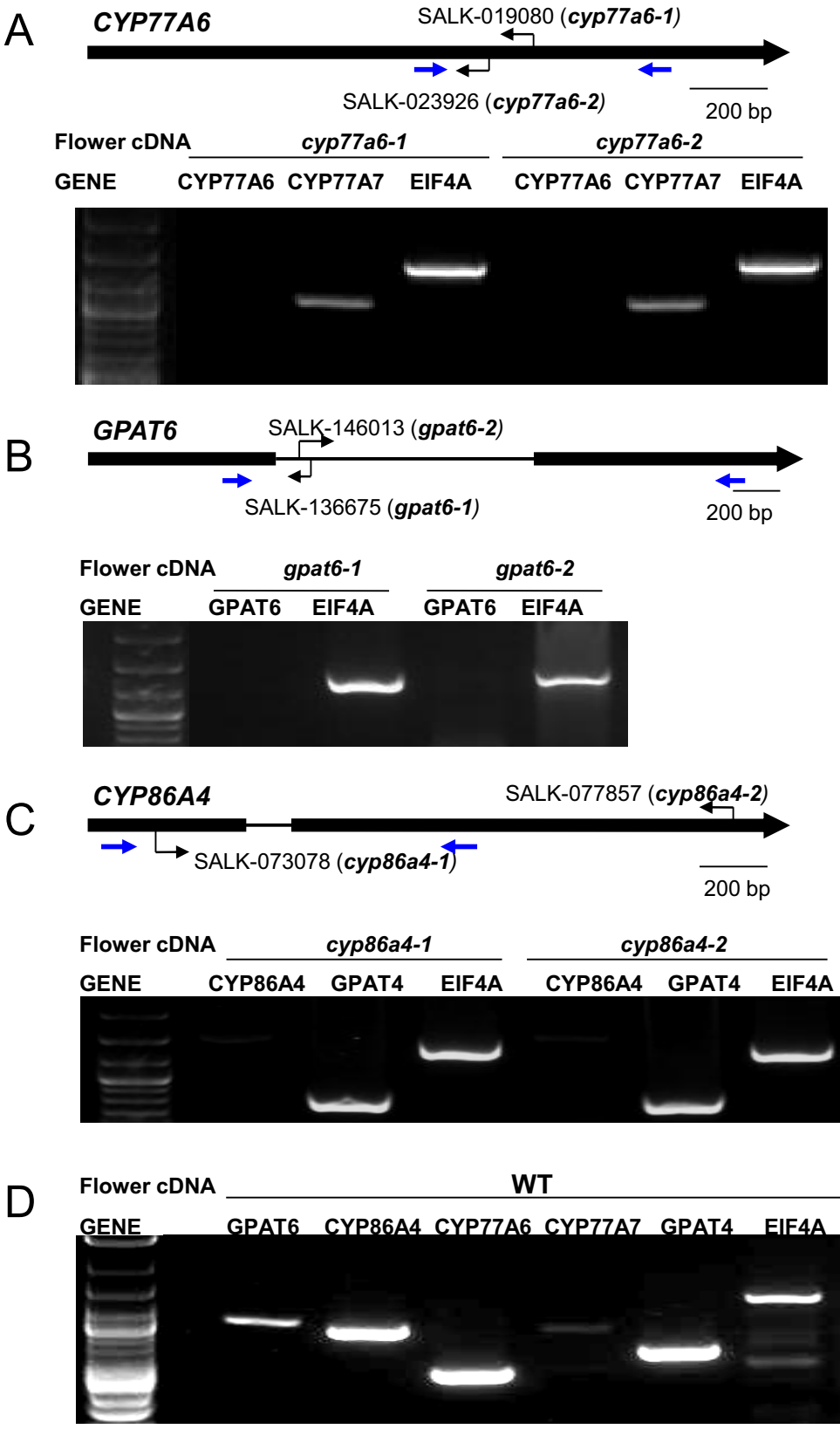

Fig. S1. Genomic organization of the T-DNA insertional mutants for *CYP77A6* (*A*), *GPAT6* (*B*), and *CYP86A4* (*C*) and confirmation of their gene silencing by RT-PCR. Expression of all three genes in WT inflorescences is shown in *D*. The *eIF4A1* (At3g13920) gene was used as load control. Black arrows indicate direction of T-DNA left border primers. Blue arrows indicate the position of the primers used for RT-PCR. Note that two cutin-related genes, *GPAT4* (At1g01610) and potentially *CYP77A7* (At3g10560), are located adjacent to *CYP86A4* (At1g01600) and *CYP77A6* (At3g10570), respectively. To rule out the possibility that the observed phenotype is due to mutation in either of these genes (*GPAT4* and *CYP77A7*), RT-PCR was also used to test the expression of these genes in the mutant plants *cyp86a4* and *cyp77a6*, respectively.

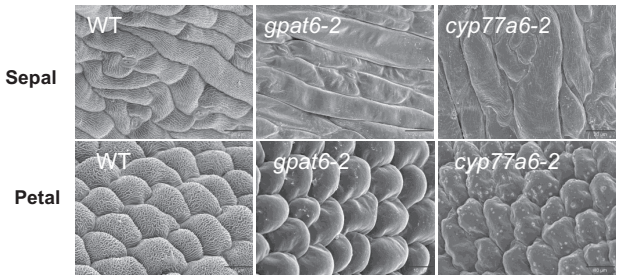

Fig. S2. TEM observation of petal and sepal surfaces in WT and mutants (*gpat6-2* and *cyp77a6-2*). (Scale bars: 20 μm for sepals and 10 μm for petals.)

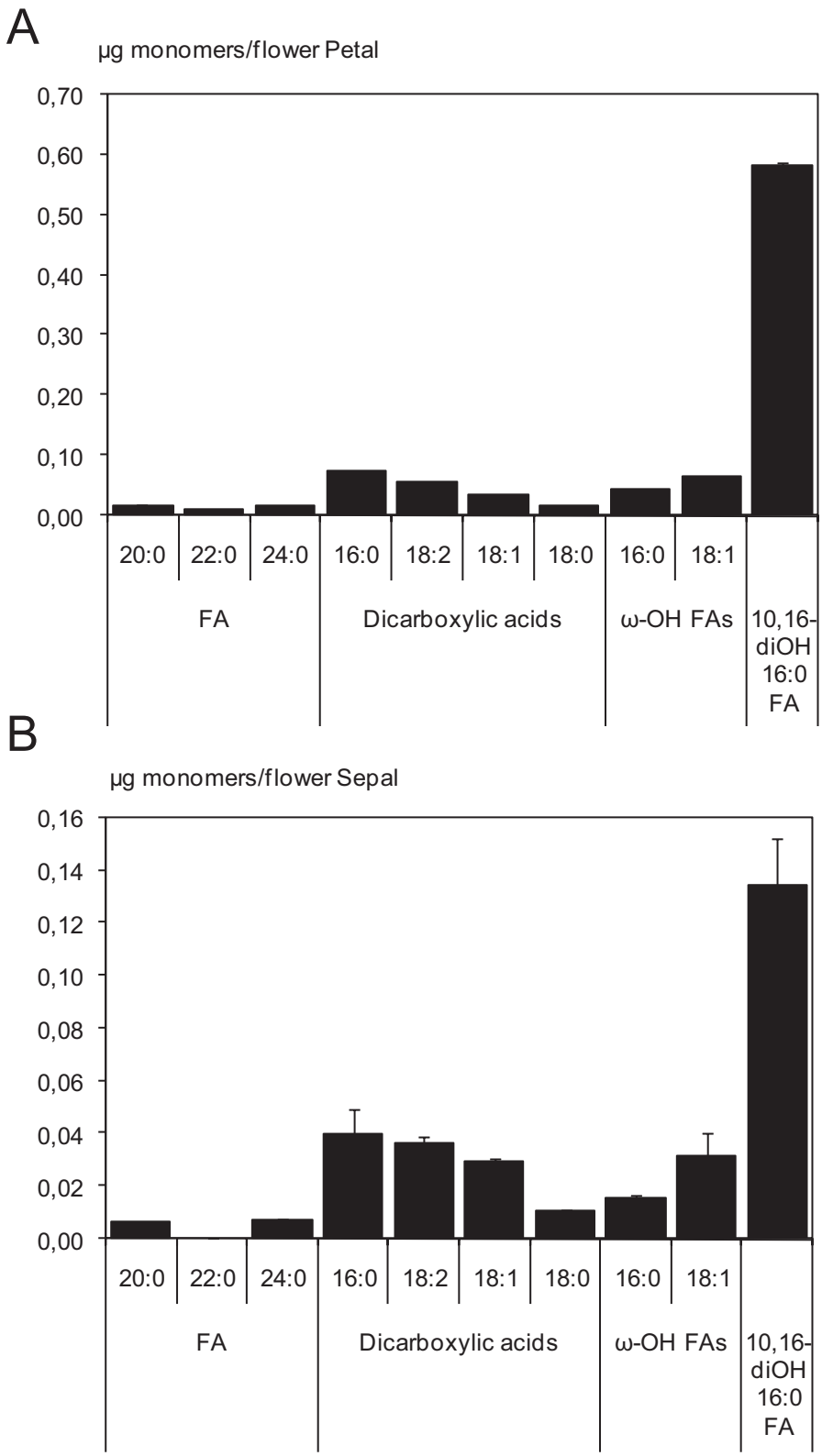

Fig. S3. Polyester monomer content in petals (*A*) and sepals (*B*) of mature WT flowers. Data are mean with SE ($n = 3$). DCA, α, ω-dicarboxylic acids; FA, fatty acids.

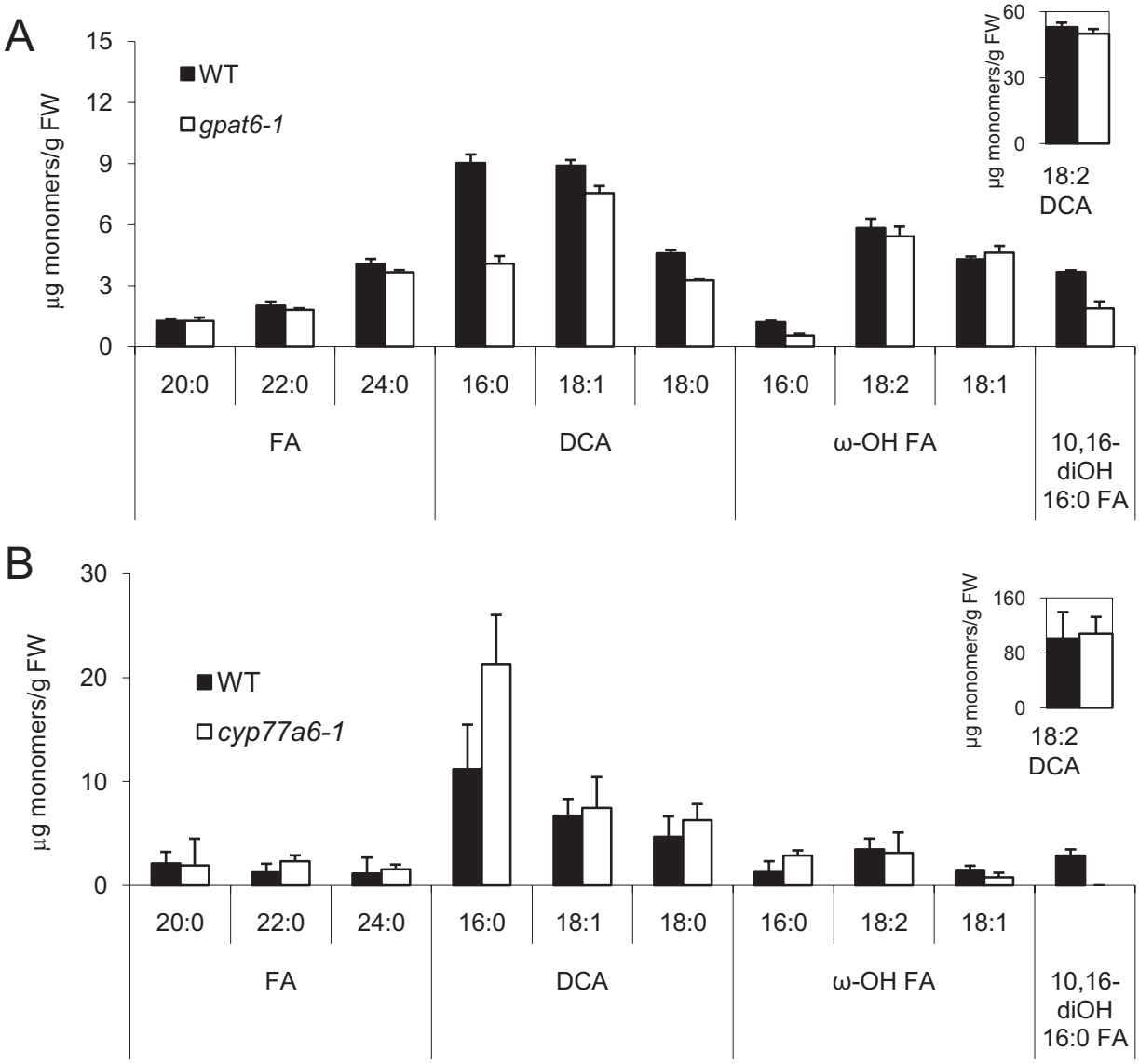

Fig. S4. Polyester monomer analysis for the 4-week-old rosette leaves of WT and *gpat6–1* mutant (*A*) and for 6-week-old stems of WT and *cyp77a6–1* mutant (*B*). Data are mean with 95% CI ($n = 3$). DCA, α, ω-dicarboxylic acids; FA, fatty acids.

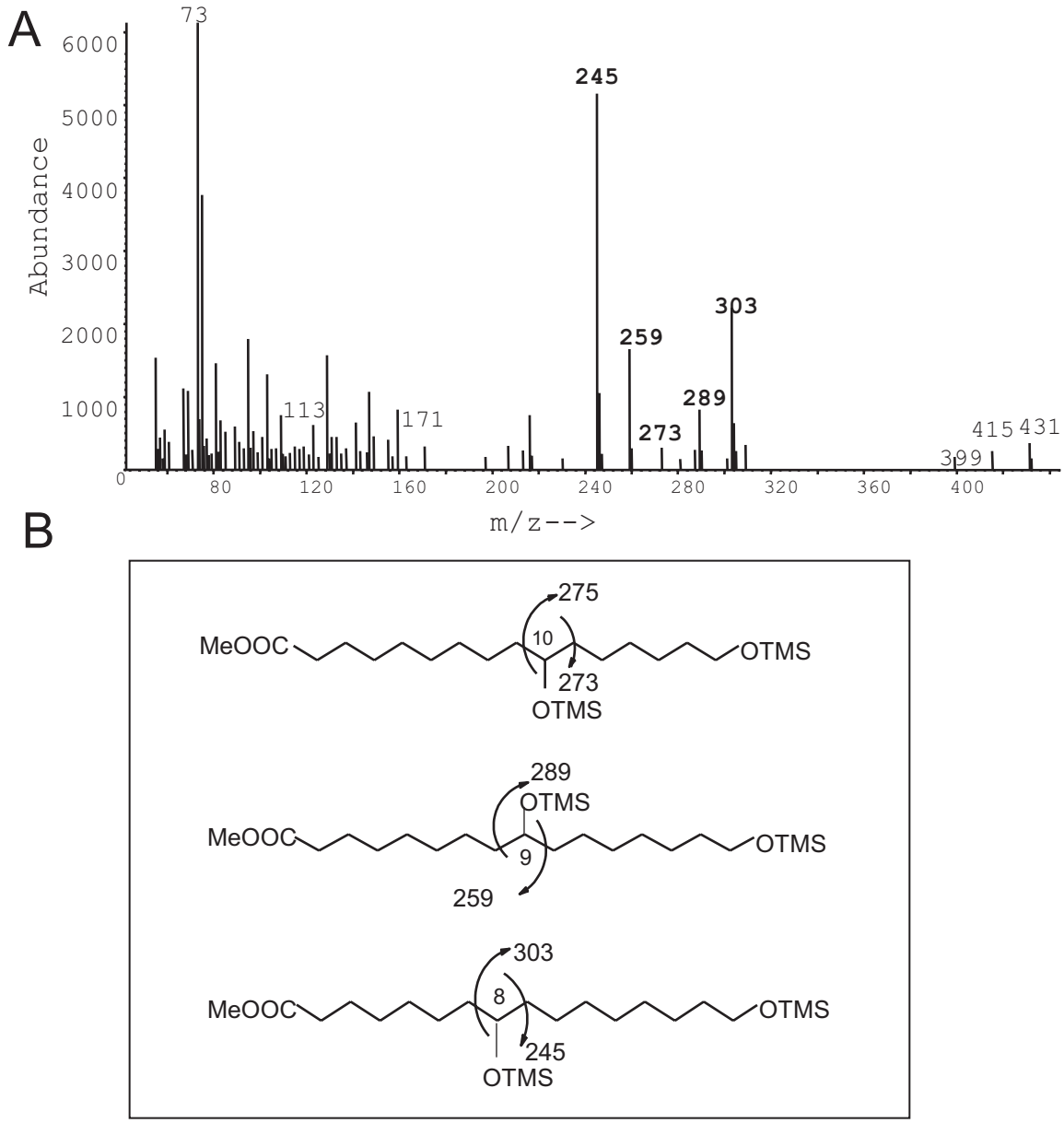

Fig. S5. Mass spectrum of methyl esters and TMS ethers of dihydroxypalmitates (*A*) and their chemical fragmentation patterns for all three positional isomers (*B*).

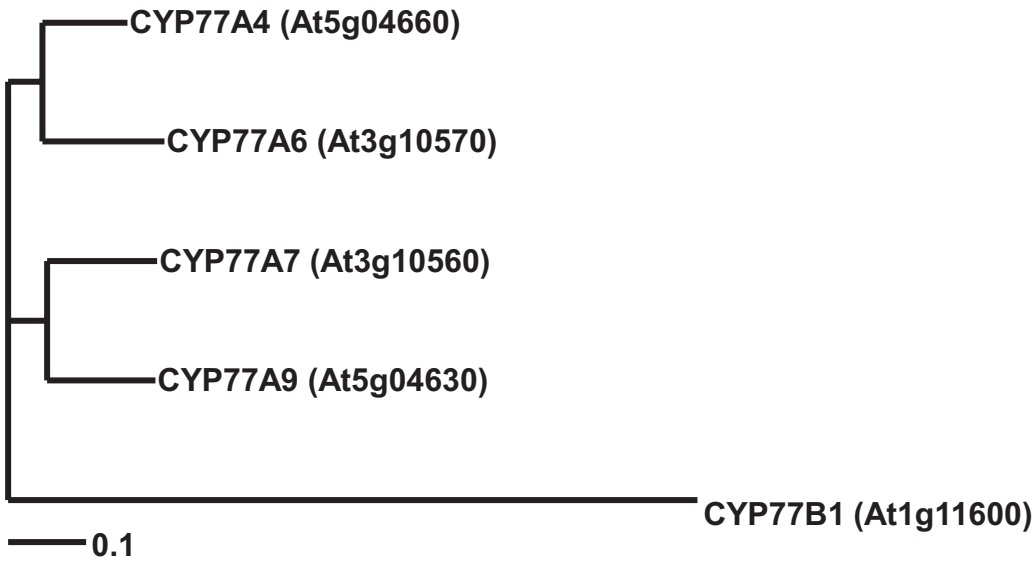

Fig. S6. Gene tree of the CYP77 family in *Arabidopsis*. Protein sequences were aligned using clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and the tree was built using the neighbor-joining method with the options correction of distances and gap ignored.

Table S1. Primer sequences

Primer name	Primer sequences (5′–3′)
T-DNA screening primers	
LBa1	TGGTTCACGTAGTGGGCCATCG
GPAT6-tF	CGTGACGTCGTTTTGAGAGA
GPAT6-tR	GTTGTAACGGGCGATACGTT
CYP86A4-tF	CACCGATAACAACGGAGGTT
CYP86A4-tR	TCTCCTAAGCTTCGGCTCAA
CYP77A6-tF	TGATTCAACGTGGACCAATG
CYP77A6-tR	CCGTGACGGAATGAGTCAAC
RT-PCR primers	
EIF4A-F	CCAGAAGGCACACAGTTTGATGCA
EIF4A-R	TCATCATCACGGGTCACGAAATTG
CYP86A4-F2	GGAAATATCCAATGCCATGC
CYP86A4-R1	GGGTTCCACTTTCTTGCTGA
GPAT4-F	CCGTGGATTGATCCTTCTTC
GPAT4-R	GGTTGAAGTAGACGCGGAAG
CYP77A6-rtF	AGAAGCCGGGGACTGATAA
CYP77A6-rtR	GGTTATGTCCGCTTCCTCCTTA
CYP77A7-rtF	ATCAGTTGATGACGGCGATA
CYP77A7-rtR	CTCTGCGGTGGATAGGCTAA
GPAT6-rtF	AATTATGGTCGAGCCATTCG
GPAT6-rtR	GCTTGTATCCACGTGTGGTG
Cloning primers	
GPAT6-cF	cacacCCATGGATATCCATTCTCACCTTCCA (NcoI)
GPAT6-cR	cacacACTAGTACCGATACTTTTTTCTTTCT (SpeI)
CYP77A6-cF	CCCCAGATCTATGTCTATCCTTTCGTTTCCTC (BglII)
CYP77A6-cR	CCCCGGTACCTTAGACCCTTGGCTTAACCATG (KpnI)