SUPPLEMENTAL MATERIALS

The phospholipid:diacylglycerol acyltransferase-mediated acyl-CoA-independent pathway efficiently diverts fatty acid flux from phospholipid into triacylglycerol in *Escherichia coli*

Lian Wang[a, 1], Shan Jiang[a, 1], Wen-Chao Chen[a, 1, b, c, d], Xue-Rong Zhou[e], Ting-Xuan Huang[a], Feng-Hong Huang[a, b, c, d], Xia Wan[a, b, c, d]

[a]Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan 430062, P.R. China

[b]Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture, Wuhan 430062, P.R. China

[c]Oil Crops and Lipids Process Technology National & Local Joint Engineering Laboratory, Wuhan 430062, P.R. China

[d]Hubei Key Laboratory of Lipid Chemistry and Nutrition, Wuhan 430062, P.R. China

[e]CSIRO Agriculture & Food, Canberra 2601, Australia

Corresponding author: Xia Wan, wanxia@oilcrops.cn.

[1]Lian Wang, Shan Jiang, and Wen-Chao Chen are co-first authors.

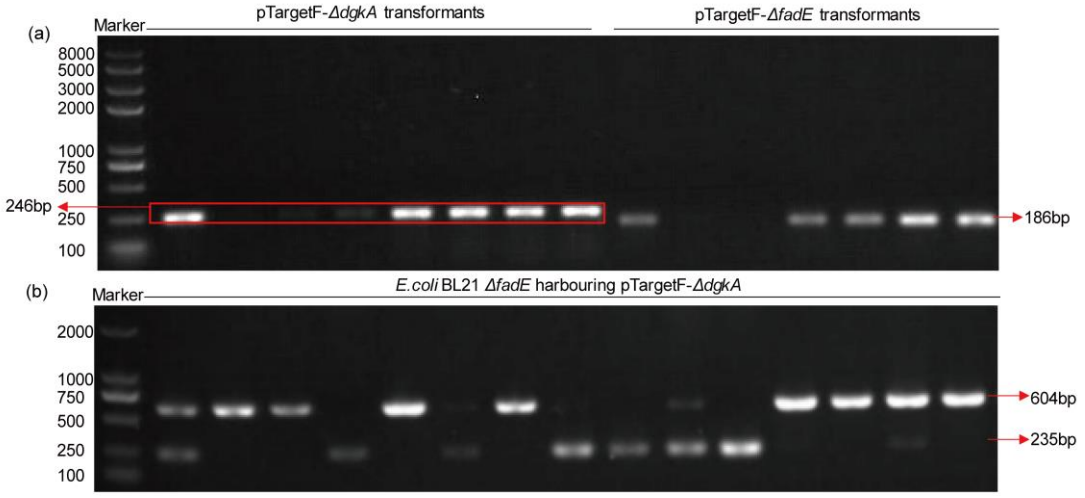

Figure S1 Confirmation of double mutant strain ΔΔBL21 by using PCR analysis. a**,** either *dgkA* or *fadE* was deleted in BL21. b, deletion of *dgkA* in BL21*ΔfadE*.to generate double mutant ΔΔBL21.

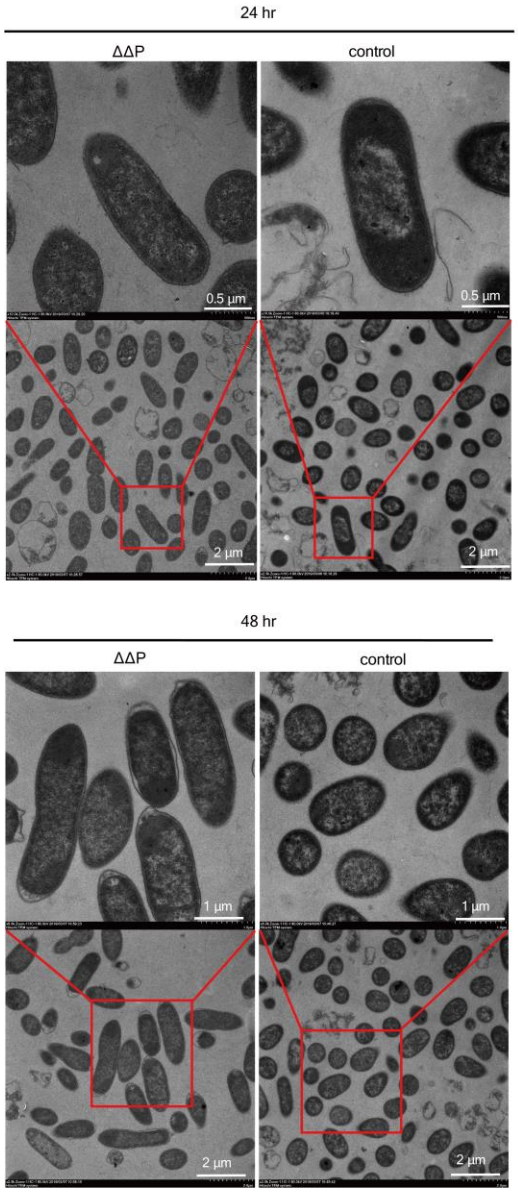

Figure S2 Observation of cells harboring either *CrPDAT* (ΔΔP) or empty vector (control) by using transmission electron microscopy. Cells were cultured in auto-induction medium with 3% glycerol at 37 °C with shaking at 200 rpm for 24 or 48 hrs.

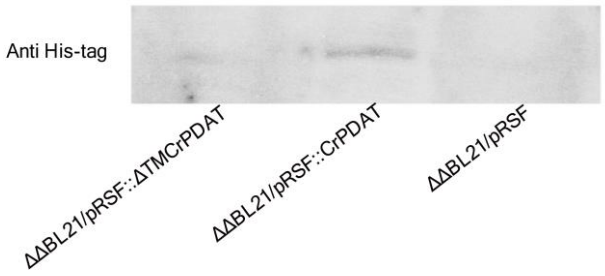

Figure S3 Western blot analysis of double mutant ΔΔBL21 harboring either full-length *CrPDAT* or truncated *ΔTMCrPDAT*. The proteins from engineered cells were blotted with anti His-tag monoclonal antibody. ΔΔBL21 harboring empty vector was used as negative control.

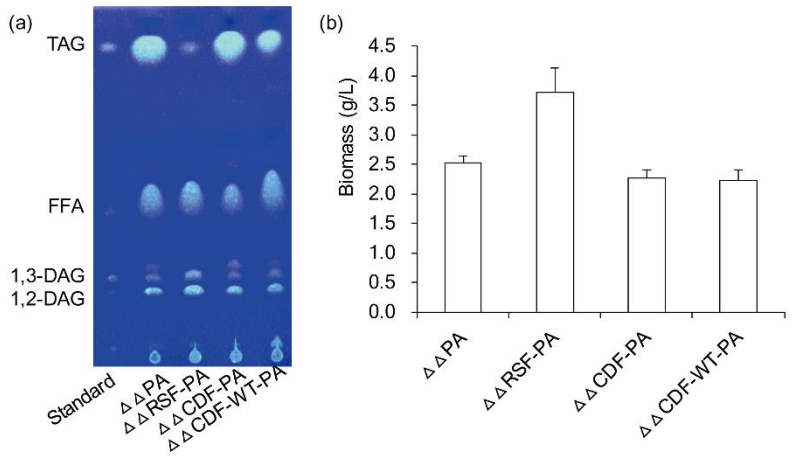

Figure S4 Change of vector or ribosome binding site (RBS) greatly affects TAG production. a, Thin-layer chromatography (TLC) result of neutral lipid profile. b, Biomass from different recombinant cells. Cells were cultured in auto-induction medium with 3% glycerol at 37 °C with shaking at 200 rpm for 48 hrs. Lipids extracted from 10 mg of dried cells. All data are the mean ± standard deviations from triplicate samples.

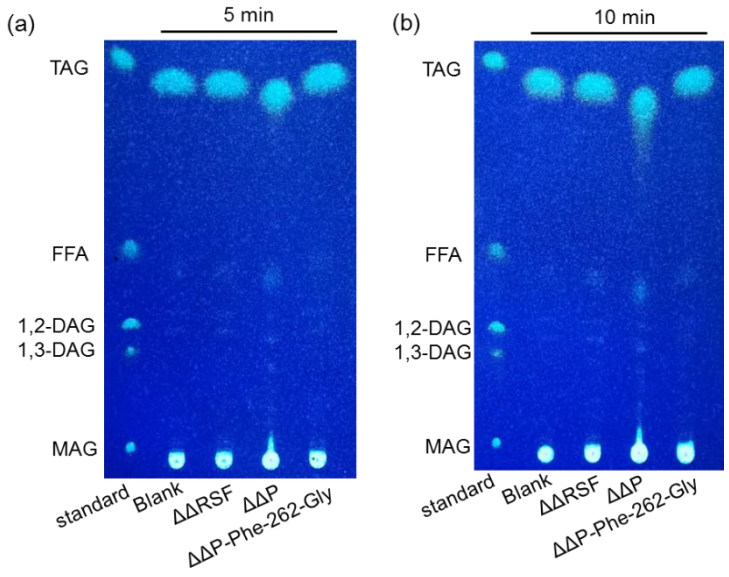

Figure S5 *In vitro* enzyme assay for detecting TAG lipase activity of CrPDAT and its mutant Phe-262-Gly. Lipids extracted from reaction mixture for 5 min (a) and 10 min (b) were analyzed by thin-layer chromatography (TLC). Blank: reaction mixture without enzyme; ΔΔRSF: ΔΔBL21 harboring empty vector pRSFDuet-1 as a negative control; ΔΔP: ΔΔBL21 harboring CrPDAT; ΔΔP-Phe-262-Gly: ΔΔBL21 harboring CrPDAT mutant Phe-262-Gly.

Table S1 List of codon optimized sequences for CrPDAT, ScPDAT and RcPDAT.

Name	Codon optimized sequence
CrPDAT	atgaccacaccgaccaaaggtaatagcaatgcacgtcagcgtaaaaaaggtggcaccagcgcagaag caagcgcagcaaccccgagcaaagcaaaaccgggtcgtgatcataatgttcatgcgaccccgagccat agccattcacatagccatagtcagagccagcagcatcgtcagggtccgcatgcagcacagccgaaaag cgaacgtcgtctggttctgtggctggcagcagccggtgttgttctgctgccgctggtgctgctgcctccgg cactgatggttgaagaaggtccggttgcagaactgctgcatgatagcctgggtctgcctcgtctgaatgca tatctgcctccgctgccgagcattgttgcactggatagcctgctggcgagcgcaggttttgcagttccgag cagcgcactgaatggcaccagtgccgctgcagcagcagcaaatagcagcgcatataatggtctgctgg cactgtttggtgttaccggtgatatggcaagtgcaggtatggatggtctgaccagccgtaccgttaccgca gcgcagcagctgcgtgcatttctggcacagctgcctgcactgagcattccaggtggtccggcagcgacc ggtggtggtcctgctggtgctggtgctggtgccggtgctggtgcaggcgctgaagttaatgcaaccgca gccgcagatgaagcagcaggtctgcgtccgggtcagctgatggcacgtcgtggttatcgtgcaaaacat ccggttgttattgttccgggttttgttaccagcggtctggaactgtggcgtggtctgccgtgtggtcagcgtt attttcgtcagcgcatgtggggcaccctggcaatggttcaggcatttctgaccgatgcagcatgttggtttc gtcacatggaactggataccgttagtggtctggatccggaaggtattaaactgcgtgcagcactgggttta gaagcagttgattatttcattcaaggctattgggtttggggtaaactggttgaagcactggccgatgttggtt atgatagcaatagcctggttagcatgccgtatgattggcgtctggcagttccgctgctggaagaacgtgat ggttattatacccgtctgcgtcgtaccattgaacagctggttgaactgaccggtgaacgtgttgttgtgacca gccatagttatggtgaaaatgtttttcgtgccttcatgcattgggttgaagccgcagctgccgaagaagagg aaggcggtaaacagcctcgtagtggtggtggtagcggtcattcaggtggtggttgggttgatcgtcatatt gcaagcaccattaacattgcaggcaccagcctgggtgttccgaaaagtgttagcgcactgctgagcggt gaaacccgtgataccgcacagctgggtgccctggcaggttttctgaccagtaatatggttccgcgtgccg cacgtacccgtgtttggcgtagctggggtgcaagctatgcaatgctgccggttggtggccctggtgtgtg gggtaatgcaagctgggcacctgatgatacaccggaaatgcgtgcaaatcgtcgcacctttggtagcatg gttagcctgtggcctcataattggcaggccctgatggcagctgcaccgcagcaacagcgcgaacagctg agccagctggtagccgaagttagccaggatgttgatgcaggtagcggtagcggttctggtagtggttcag gatcaggttctggcagcggtagtggcagcggctcaggctctggctcaggtagtggtgaagatgcagtta atggtacaggtggtgcaggtagtgccggtggggctgcactggctccggcagaagcagcggcagttggt acagcaggcggtggtgataatgccacagcagccaccgcagttaatagcaccaccgcaagcaatagtac cggtagcgcaggcaccggtagcagcggttccggtggtagcagcagctcaaaagcaattgcagccattg cagataccatcaaaaaactggtaggtggtggcgttgatgccgcagaatttctgaatagcgatcatgtgacc cgtctggatgttagcggttttattgcactgctgcgtgaagttggcggtccgctggttagcgcaaatattgcac agtggggtgcgctgcagttacctgcagaagaggcagccgaagcagttcgtaaacgtgccgcagcagc gagcgatcgtgcagctcctgccgcagccggttctccggcagcacaggctgttggtggtgccgatcgtgtt aaagatagcactgccggtggtgcgggtgcagcaggtattggtcgtggtggttcatcagcaggttcaggtc gtgttgccctgtttccggatgcaacccgtacaccgctgccgtcagcaccgaaaaccaccatggtttgtctg tatggtgttggcctgccgaccgaacgcggttatcattatctgcgtcctccgcctccgaccggtgcagccgc aaccgcaacagccgcagcggcaaatgcaccggcaggtaatgcggctgcagcccctgcagcagatgca cagcctcctccacaagccgctggcggaggtgatggtgatggcatggcaaccaccgatggtacagcgga agccgatgcagaacgtggtgttccgcctcgtggtgcaaccgcctcaggtgaagaggcaaatcctgatgc

	tggcggtgatgcaagcagtggttggatgattatgaaagatgttacccaggcacagagcgcactggatgtt ggtgttcatattagcgacggtgatggtacggttcctctgctgagtctgggtctgatgtgtcgcggtggttgg cgtgaagcaggtcatctgaatccgggtgcaatgcgtgttgttacccgtgaatataaacataaagcagtgag catgctgcaggatgcacgtggcggtccagcagcagccgcacatattgatattctgggtaatgatgccgttc tgcgtgatgtgattaccgttgttgcaggtcgtgcagatgaactggcagatattgttgttagcgatattgatcgt attgcggcagccgttgattgggcagcgctggcagcctaa
ScPDAT	atgggcaccctgtttcgtcgtaatgttcagaatcagaaaagcgatagcgacgaaaataacaaaggtggta gcgtgcataataaacgtgaaagccgtaaccatatccatcatcagcaaggtctgggtcataaacgtcgtcgt ggtattagcggtagcgcaaaacgtaatgaacgtggcaaagattttgatcgcaaacgtgatggtaatggtc gtaaacgttggcgtgatagccgtcgtctgatttttatcctgggtgcatttctgggtgttctgctgccgtttagct ttggtgcatatcatgttcataattcggatagcgacctgttcgataactttgtgaatttcgatagcctgaaagtgt atctggatgattggaaagatgttctgccgcagggtattagcagctttattgatgatattcaggcaggcaatta tagcaccagcagcctggatgatctgagcgaaaattttgcagttggtaaacagctgctgcgcgattataaca ttgaagcaaaacatccggttgttatggttccgggtgttattagcaccggtattgaaagctggggtgtgattg gtgatgatgaatgtgatagcagcgcacattttcgtaaacgcctgtggggtagcttttatatgctgcgtaccat ggttatggataaagtttgttggctgaaacacgttatgctggatccggaaaccggtctggatccgcctaatttt accctgcgtgccgcacagggttttgaaagcaccgattatttcattgccggttattggatttggaacaaggtgt ttcagaatctgggcgttattggttatgaaccgaataaaatgaccagcgcagcctatgattggcgtctggcat atctggatctggaacgtcgtgatcgttatttcaccaaactgaaagagcagatcgaactgtttcatcagctga gcggtgaaaaagtttgtctgattggtcatagcatgggcagccagattatcttctattttatgaaatgggtcga agccgaaggtccgctgtatggcaatggtggtcgtggttgggttaatgaacatatcgatagctttattaacgc agcaggcaccctgctgggtgcaccgaaagcagttccggcactgattagtggtgaaatgaaagataccat ccagctgaataccctggcaatgtatggtctggaaaaattctttagccgtatcgaacgtgttaaaatgctgca gacctggggtggtattccgagcatgctgccgaaaggtgaagaagttatttggggagatatgaaaagcag cagcgaagatgcactgaataacaataccgatacctatggcaacttcattcgctttgaacgtaataccagtga cgcctttaacaaaaacctgaccatgaaagatgcgatcaatatgaccctgagcattagtccggaatggctgc agcgtcgtgtgcatgaacagtatagttttggttatagcaaaaacgaagaggaactgcgcaaaaatgaact gcatcataaacattggagcaacccgatggaagttccgctgccggaagcaccgcatatgaaaatctattgt atttacggcgtgaacaatccgaccgaacgtgcatatgtttataaagaagaggacgatagctcagcactga atctgaccattgattatgaaagcaaacagccggtttttctgaccgaaggtgatggcaccgttccgctggttg cacatagcatgtgtcataaatgggcacagggtgcaagcccgtataatccggcaggtattaatgttaccatc gtggaaatgaaacatcagccggatcgttttgatattcgtggtggtgcaaaaagcgcagaacatgttgatatt ctgggtagtgcagaactgaacgattacattctgaaaattgcaagcggtaatggtgatctggttgaaccgcg tcagctgtcaaatctgagccagtgggttagccagatgccgtttccgatg
RcPDAT	atgtctatcctgcgtcgtcgtctgcgtgttcagaactcttctcagatcgaagctgacaacgacgaaaaagaa aaagaaaaacgtaaacgtaaaaaagaaatcaaaaaatggcgttgcgttgacaactgctgctggttcatcg gtttcatctgctctatgtggtggttcctgctgttcctgtacaacgctatgccggcttctttcccgcagtacgtta ccgaagctatcaccggtccgatgccggacccgccgggtgttaaactgcgtaaagaaggtctgaccgtta aacatccagttgtcttcgtcccgggtatcgttaccggtggtctggaactgtgggaaggtcaccagtgcgct gacggtctgttccgtaaacgtctgtggggtggtaccttcggtgacctgtacaaacgtccgctgtgctgggtt

	gaacacatgtctctggacaacgaaaccggtctggacccgccgggtatccgtgttcgttctgtttctggtctg gttgctgctgactacttcgctgctggttacttcgtttgggctgttctgatcgctaacctggctcgtctgggttac gaagaaaaaaacatgtacatggctgcttacgactggcgtctcagcttccagaacaccgagatccgtgacc agtctctgtctcgtatcaaatctaacatcgaactgatggttgctaccaacggtggtaacaaagttgttgttctg ccgcactctatgggtgttccgtacttcctgcacttcatgaaatgggttgaagctccggctccgatgggtggt ggtggtggtccggactggtgcgctaaacacatcaaagctgttatcaacatcggtggtccgttcctgggtgt tccgaaagctatctcttctctgttctctaacgaaggtcgtgacatcgctgctgctcgtgctttcgctccgggtt tcctggacaaagacgttttcggtctgcagaccttccagcacgctatgcgtctgacccgtacctgggactct accatgtctatgatcccgaaaggtggtgaaaccatctggggtggtctggactggtctccggaaggtgttta caactgcggttctaacaccccgaaaaacaacaacacccagaccgctggtcagaccggtaaaggtacctc ttctttcaccgaaggtgttaactacggtcgtatcatctctttcggtaaagacgttgctgaactgcactcttctaa aatcgaccgtatcgacttccgtgacgctgttaaaggtaaccgtgttgctaacaactgcgacatctggaccg aataccaggaaatgggtatcggtggtatcaaagctgttgctgactacaaagtttacaccgctggttctgttat cgacctgctgcacttcgttgctccgaaactgatggctcgtggtgacgctcacttctctcacggtatcgctga caacctggacgacccgaaatacgaacactacaaatactggtctaacccgctggaaacccgtctgccgga cgctccggaaatggaactgtactctatgtacggtatcggtatcccgaccgaacgtgcttacatctacaaact gaccctgacctctgaatgcgctatcccgttccagatcgacacctctgttaccggtggttctgaaaactcttg cctgaaagacggtaccctgaacgttaacggtgacgaaaccgttccggttctctcggctggcttcatgttcg ctaagggttggcgtggtaaaacccgtttcaacccgtctggtatccacacctacatccgtgaatacaaccac gctccgccggctaacctgctggaaggtcgtggtacccagtctggtgctcacgttgacatcctgggtaactt cgctctgatcgaagacgttctgcgtatcgctgctggtgctcgtggtgaagacctgggtggtgaccgtgttta ctctgacatcttcaaatggtctgaaaaaatcaacctgccgctg

Table S2 Information on PDAT proteins used in this study.

Protein name	Organism	Number of amino acids	Database accession number
CoPDAT	*Camellia oleifera*	673	AKI88004.1
CsPDAT	*Camelina sativa*	671	AQU71887.1
AtPDAT	*Arabidopsis thaliana*	671	AED91921.1
RcPDAT	*Ricinus communis*	685	ADK92410.1
EsPDAT	*Eutrema salsugineum*	671	XP_006399859.1
BnPDAT	*Brassica napus*	660	XP_022568870.1
GhPDAT	*Gossypium hirsutum*	697	XP_016733508.1
CrPDAT	*Chlamydomonas reinhardtii*	1040	AFB73928.1
PpPDAT	*Pseudochlorella pringsheimii*	649	AOS88520.1
LiPDAT	*Lobosphaera incisa*	691	ANH71127.1
PhPDAT	*Plasmopara halstedii*	670	CEG44972.1
FsoPDAT	*Fistulifera solaris*	702	GAX24664.1
OtPDAT	*Ostreococcus tauri*	665	XP_003081310.1
TpPDAT	*Thalassiosira pseudonana*	468	XP_002286433.1
FsPDAT	*Fistulifera solaris*	703	GAX19192.1
DsPDAT	*Diplodia seriata*	634	KKY28315.1
AcPDAT	*Aspergillus campestris*	621	PKY08229.1
CaPDAT	*Coniosporium apollinis*	641	XP_007779229.1
DcPDAT	*Diplodia corticola*	634	XP_020133357.1
AclPDAT	*Aspergillus clavatus*	632	XP_001270995.1
AcaPDAT	*Aspergillus candidus*	621	PLB40870.1
PcPDAT	*Pochonia chlamydosporia*	623	XP_022285424.1
AbPDAT	*Aspergillus bombycis*	625	XP_022393766.1
NpPDAT	*Neofusicoccum parvum*	637	XP_007582622.1
PsPDAT	*Penicillium subrubescens*	633	OKO99574.1
ApPDAT	*Aureobasidium pullulans*	636	KEQ89966.1
AlPDAT	*Aspergillus lentulus*	621	GAQ04464.1
PgPDAT	*Penicillium griseofulvum*	622	KXG48669.1
BsPDAT	*Byssochlamys spectabilis*	630	GAD93278.1
ArPDAT	*Aspergillus rambellii*	630	KKK27008.1
SpPDAT	*Schizosaccharomyces pombe*	632	NP_596330.2
SoPDAT	*Schizosaccharomyces octosporus*	632	XP_013018030.1
ZbPDAT	*Zygosaccharomyces bailii*	645	CDH09035.1
LmPDAT	*Lachancea meyersii*	642	SCV03918.1
ScPDAT	*Saccharomyces cerevisiae*	661	NP_014405.1
OpPDAT	*Ogataea parapolymorpha*	659	XP_013933810.1
KmPDAT	*Kluyveromyces marxianus*	640	BAP73190.1

Western blot analysis

Engineered cells harbouring either *CrPDAT or ΔTM-CrPDAT* were cultivated at 37°C in LB medium supplemented with kanamycin until their OD_{600} reached 0.6. Then cells were cultured at 30°C for 4 hr after addition of 0.5 mM isopropyl ß-D-thiogalactoside (IPTG). Cells were collected by centrifugation, resuspended in sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min. Protein samples were next separated on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking non-specific binding using 5% skim milk for 1 hr, the blots were incubated with anti His-tag (His Mouse Mab, Promoter) at 4°C overnight. Membranes were washed three times with TTBS buffer (20mM Tris-HCl, 150mM NaCl, 0.1% Tween 20, pH 7.6) and incubated with an appropriate secondary antibody (HRP-Goat Anti Mouse IgG(H+L), Merck) for 1 hr at room temperature. After being washed three times with TTBS, direct chemiluminescence (SuperSignal™, Thermo Scientific) imaging of the blots was performed using the ChemiDox XRS (BioRad) imaging system. Engineered cells harbouring empty vector pRSFDuet-1 were used as control.

***In vitro* assay for detecting TAG lipase activity**

For the collection of recombinant PDAT protein, ΔΔBL21 harboring pRSF::*CrPDAT* or pRSF::*CrPDAT*-Phe-262-Gly was incubated into LB medium containing 50 mg/L kanamycin and incubated at 37°C with shaking at 200 rpm overnight. ΔΔBL21 harboring the empty vector pRSFDuet-1 was used as a negative control for this assay. The seed cultures were subsequently incubated into 50 mL LB containing 50 mg/L kanamycin in 250 mL flask at 37°C with shaking at 200 rpm until the OD_{600} reached 0.7. IPTG (Isopropyl-*β*-D-thiogalactopyranoside) was added into the culture at a final concentration of 0.1 mM, and the cells was induced at 15°C for another 20 hrs with shaking at 200 rpm. Cells were harvested by centrifugation at 6000 rpm for 8 min, and then washed with 50 mM Tris-HCl buffer (pH 7.4) twice. After transferred into 2 mL tubes, cells were lysed by ultrasonic disruption at 20 Hz for 10 min (time for each treatment was 3 min, with a 2 min interval). The supernatants were collected by centrifugation at 4°C.

To investigate the TAG lipase activity of CrPDAT and its mutant Phe-262-Gly, 20 μL of crude enzymes in 50 mM potassium phosphate buffer (pH 7.4) were added into a 400 μL of reaction mixture containing 339 μL PBS buffer, 1 μL tween20, and 40 μL C15:0-TAG. The mixture was incubated at 30°C for 10 min. The reaction was terminated by 375 μL methanol and chloroform with ratio of 2:1 (v/v). Lipids were extracted from the mixture by adding 375 μL mixture of methanol and chloroform (1:1, v/v) into the reaction. The extracted lipids were next analyzed by TLC as mentioned in Materials and Methods.