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lactolipids [12–14].
corporated fatty acid at the sn-2 position
plastidic pathway-derived galactolipids (16:X) [15,16].

The ER is also the site of triacylglycerol (TAG) biosynthesis. TAG have an important function as a storage compound in seeds [17]. The acylation of DAG to TAG is either catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT) or by phospholipid:diacylglycerol acyltransferase (PDAT) using acyl-CoA or PC as acyl-donor, respectively [18–23]. Mutant analysis in *Arabidopsis thaliana dgat1* and *dgat1/pdat1* knock-out lines demonstrated, that DGAT1 and PDAT1 are responsible for the majority of seed TAG biosynthesis [17–21].

Furthermore, labeling experiments in different plant species showed that acyl-editing is also important for major seed TAG formation [24–28]. Newly synthesized fatty acids are incorporated into PC by aycl-CoA:lysophosphatidylcholine acyltransferases (LPCAT) for desaturation by oleate and linoleate desaturases, FAD2 and FAD3 (fatty acid desaturase), respectively [29,30] and polyunsaturated fatty acids (PUFA) are released from PC to the acyl-CoA pool [31,32], possibly catalyzed by phospholipase A (PLA) and LPCAT [33–36].

Upon Pi starvation, plants utilize membrane phospholipids as an internal Pi source. To maintain membrane functionality phospholipids are replaced by galactolipids. Two pathways are considered to mainly dephosphorylate phospholipids under Pi deficient conditions: a one-step reaction by non-specific phospholipase C (nsPLC: NPC4 and NPC5) and a two-step reaction by phospholipase D (PLDζ1 and PLDζ2) together with PA phosphohydrolase (PAH1 and PAH2) [37–44]. Especially DGDG serves as the main surrogate for most decreasing phospholipids in leaves and roots [42,45], while SQDG replaces PG in the chloroplast [46,47]. Furthermore, analyses mainly in *A. thaliana* showed that phosphate deprivation led to increased transcriptional abundance of MGDG synthase (MGD2/MGD3: [48–50]), DGDG synthase (DGD1/DGD2: [51,52]), UDP-glucose pyrophosphorylase 3 (UGP3: [53]) and UDP-sulfoquinovose synthase 1 and 2 (SQD1/SQD2: [46,54]). Also different microarray and RNA-Seq based methods in *A. thaliana*, potato and rice supported existing models of the membrane lipid remodeling upon Pi deficient conditions in plants [38,55–59].

Recently, it was reported that Pi depletion also led to an accumulation of TAG in *A. thaliana* shoots and roots [60,61] and soybean leaves [62]. But still little is known about TAG accumulation under Pi deficient conditions and the contribution of the different diacylglycerol acyltransferases in vegetative tissue, making it important to obtain a detailed understanding of stress-induced changes in TAG metabolism.

We employed mass spectrometry and RNA-Seq based methods to investigate the acclimation of lipid metabolism in *S. lycopersicum* leaves and roots to Pi-deficient conditions. The results suggest that the availability and composition of the acyl-CoA pool and ER-derived precursors determine the flux of degraded phospholipids toward triacylglycerol or galactolipid synthesis.

2. Material und methods

2.1. Plant growth conditions

Solanum lycopersicum cv. M82 seeds were treated with 2.7% NaClO and finally sowed on ½ MS medium plates (pH 5.7) containing 0.8% (w/v) plant agar. Seeds were germinated in a phytotron with 16/8 h dark/light regime and a light intensity of 250 µmol photons m^{-2} s^{-1} at 22 °C/18 °C. After 12 days, seedlings were transferred to hydroponic boxes containing Hoagland medium [63] and cultivated in a green house chamber in 12/12 dark/light cycle with 30%/60% humidity and drogenphosphate was replaced by equimolar amounts of ammoniumsulfate maintaining the same cation concentration. Leaves or roots were harvested and directly frozen into liquid nitrogen and stored at −75 °C. For all lipidomic and transcriptomic analysis the same plant material was used.

2.2. Lipid extraction

Lipid extraction was carried out by the method modified from [64]. Plant material was ground with mortar and pistil under liquid nitrogen and quantitatively extracted with 7.5 ml ice cold $CHCl_3$:MeOH (2:1, v/v) in glass grinding tubes containing 1 volume of ceramic beads (3.0–3.3 mm diameter, Soilgene). After washing with 0.9% NaCl solution, the $CHCl_3$ phase was dried and re-suspended in $CHCl_3$.

2.3. Transesterification of fatty acids and GC–MS analysis

Lipid extracts were transmethylated in 2 ml 0.5 M H_2SO_4 in MeOH at 80 °C for 1 h. 1 mM pentadecanoic acid (Sigma Aldrich) was used as internal standard. Fatty acid methyl esters were extracted with 3 ml hexane and analyzed by GC–MS(Q) (Agilent 7890A) with an Optima 5 MS column (30 m × 250 nm × 0.25 µm film thickness; Machery and Nagel). Helium was used as the carrier gas with a constant flow rate of 1 ml/min. Each sample was split injected with 1 µl (1:10, T = 275 °C). Oven ramp was set as follows: 120 °C hold for 2 min, 150 °C (10 °C/min), 186 °C (3 °C/min), 195 °C (1 °C/min), 207 °C (3 °C/min), 220 °C (1 °C/min) and finally up to 270 °C (3 °C/min). MS source was set to 70 eV. Fatty acid methyl esters were identified by retention time and *m/z* using ChemStation Software (Agilent Technologies) and relatively quantified with determined adjustment factors for Agilent 7890A. The student's *t*-test was used to determine statistical significance.

2.4. Phospholipid and galactolipid species analysis by mass spectrometry

The composition of phospholipids and galactolipids was analyzed by direct infusion electrospray ionization tandem mass spectrometry as described in [65,66] with minor modifications. Total lipid extracts were dried and resuspended in $CHCl_3$:MeOH:300 mM NH_4-Ac (300:665:35, v/v/v). For each phospholipid two internal lipid standards (Avanti Polar Lipids) were added and one galactolipid/sulfolipid standard each (Matreya) in defined concentrations (Suppl. Table B14). Samples were continuously infused into triple quadrupole mass spectrometer (6420 Triple Quadrupole MS, Agilent) by PSD/3 syringe pump (Hamilton) with a constant flow rate of 6 µl/min. Each lipid class was measured for 1 min with specific settings shown in Suppl. Table B15. Lipids were measured as $[M+H]^+$ or $[M+NH_4]^+$ ions in neutral loss (NL) or precursor ion scan (Suppl. Table B15). After correction of isotopic overlap, lipid molecular species were identified by *m/z* and relatively quantified using a correction curve determined from standards as described in [65,66]. The student's *t*-test was used to determine statistical significance.

2.5. Analysis of triacylglycerol species by mass spectrometry

Triacylglycerol lipid species analysis and TAG adjustment factor determination were carried out by electrospray ionization tandem mass spectrometry as described in [67]. 14 available TAG standards, including C48:0 (tri16:0), C48:3 (tri16:1), C51:0 (tri17:0), C54:0

$CHCl_3$:MeOH and [illegible]
As an internal standard, tri17:1 was added to a [illegible] 0.5 µM. Samples were continuously infused into ESI-MS/MS (6420 Triple Quadrupole MS, Agilent) by PSD/3 syringe pump (Hamilton) with a constant flow rate of 6 µl/min. TAG classes were measured as $[M+NH_4]^+$ ions by a series of NL scans for 1 min each (collision energy 21 V, acceleration voltage 7 V, fragmentor voltage 135 V) (Suppl. Table B17). After correction of isotopic overlap, lipid molecular species were identified by *m/z* and relative quantified using the determined adjustment factors according to [67]. The student's *t*-test was used to determine statistical significance and multiple-hypothesis correction was performed.

2.6. Thin layer chromatography

Thin layer chromatography (TLC) was performed using SIL-G-50 plates (Macherey-Nagel) for galactolipid/phospholipid and TLC Silica Gel 60 plates (Merck) for TAG analysis. The solvent system $CHCl_3$:MeOH:acetic acid:H_2O (91:30:4:4; v/v/v/v) and heptane:-diethylether:acetic acid (90:30:1; v:v:v) were selected for galactolipid/phospholipid and triacylglycerol analysis, respectively. Tripalmitin (Sigma Aldrich) was used as TAG reference substance and lipids were stained with iodine.

2.7. In silico analysis of lipid pathways

The orthologous mapping of proteins involved in lipid metabolism was performed using BLAST+ [68] against reference protein sequences of *A. thaliana* using mainly the lipid pathway databases of Li-Beisson et al. [69] and information on TAIR. Results were also compared to Mercator [70]. The e-value threshold was set to $< e^{-30}$ in order to decide whether the respective orthologous *S. lycopersicum* gene exists.

For FAD and DGAT a detailed in silico analysis was performed, using amino acid alignment and tree construction followed by the analysis of conserved amino acid motifs. Amino acid sequences were aligned using the MUSCLE algorithm [71] with default parameters. A phylogenetic tree was constructed using MEGA 6.0.5 [72] and statistically analyzed with the maximum likelihood method and 500 bootstrapped re-samplings.

2.8. Whole trancriptome cDNA library construction and sequencing

Either tomato leaves or roots were ground to powder in liquid nitrogen using mortar and pestle and total RNA was prepared using MasterPure™ Plant RNA Purification Kit (Epicentre Technologies) for leaf samples and RNeasy Plant Mini Kit (Qiagen) followed by Baseline ZERO DNase digestion (Epicentre Technologies) for root samples, following manufacturing's protocols. Total RNA amounts were quantified using Qubit RNA BR Assay Kit (Thermo Fisher Scientific) and quality controlled using 1% (w/v) agarose gel. cDNA libraries were prepared using TruSeq Stranded mRNA Sample Preparation Kit (Illumina) with 4 µg of total RNA, following manufacturing's protocol. cDNA libraries were quantified using Qubit DNA BR Assay Kit and quality was checked on a 1% (w/v) agarose gel to estimate the library size. All libraries were sequenced on the Illumina NextSeq Platform as single end reads (NextSeq 500/550 High Output v2 kit, 75 cycles) following the manufacturer's recommendations. Data can be accessed under accession number PRJEB36671 at the European Nucleotide Archive.

mitochondrial genomes. Genome sequence (*S. lycopersicum* HEINZ build 3.00) and annotation (iTAGv3.10) were downloaded from Sol Genomics Network on 21.03.2017. Chloroplast (gi|544163592|ref|NC_007898.3|) and mitochondrial (gi|209887431|gb|FJ374974.1|) genomes were obtained from the National Center for Biotechnology Information.

Mapping was performed with Tophat v2.0.14 [74] using default parameters except the provision of an annotation file (-G < gff3 file >) to assist in finding splice junctions. The functional annotation was performed using Mercator Webserver with default settings. Unique counts were quantified using HTSeq-0.6.1 with default parameters expect for –stranded = no. To normalize expression, FPKM values (fragments per kilobase per million) were calculated with cufflinks v2.2.1 with default parameters except for -G < gff3 file > to use only provided gene models. TPM values (transcripts per million) were calculated from the FPKM values.

For the differential expression analysis likelihood ratio tests for each comparison were made from the general linear models in edgeR [75]. Multiple hypothesis correction to false discovery rate (FDR) was performed with Benjamini Hochberg method [76].

2.10. Validation of RNA-Seq data by qRT-PCR

RNA-Seq results were confirmed by qRT-PCR of selected genes. Therefore, cDNA was synthesized from isolated RNA (7 days samples) using 18mer oligo(dT) primer, reversed transcribed by M-MLV Reverse Transcriptase (Promega Corporation) according to the manufacturer's instructions and quality was checked on a 1% (w/v) agarose gel. Transcript levels were analyzed using the StepOnePlus real-time PCR system (Life technologies). Primer sequences are given in Suppl. Table B18. Fragments were amplified for 40 cycles with Platinum SYBR Green qPCR SuperMix (Life Technologies). The specificities of the amplicons were checked by melting curve analysis and amplification efficiencies were determined by serial dilution of cDNA. PP2Ac1 was used as a reference gene (Solyc05g006590) (Determination of reference gene according to [77]). Fold changes in gene expression (P vs. FN) were calculated using REST© software [78].

2.11. In vitro acyltransferase activity [^{14}C]-assay

Fresh plant material of tomato leaves or roots were mixed with 1–2 volume of homogenisations buffer (0.4 M Saccharose; 0.15 M Tricin-KOH, pH 7.5; 10 mM KCl; 1 mM EDTA; 1 mM $MgCl_2$). Homogenate was filtered through 2 layers of nylon membrane (50 µm) and centrifuged at 7000*g* at 4 °C for 10 min. Supernatant was centrifuged 18,000*g* at 4 °C for 10 min followed by an additional centrifugation step at 100,000*g* at 4 °C for 1 h. The resulting pellet was re-suspended in ice-cold Bis-Tris-Propane Buffer, pH 7.5 and stored at −75 °C.

Acyltransferase activity was measured by in vitro assay containing 50 mM Bis-Tris-Propane-HCl, pH 7.5, 400 µM 1,2-dipalmitin, 200,000 dpm [1-^{14}C] 16:0 acyl-CoA (PerkinElmer) and 50 µg tomato microsomal membranes in a total volume of 100 µl. The mixture was incubated at 30 °C for 1 h and extracted with $CHCl_3$:MeOH (2:1, v:v). Boiled microsomal fraction was used as a negative control. The organic phase was separated via chromatography on a silica gel TLC plate (Silica Gel 60G, Merck) using n-heptan:diethyl ether:acetic acid (90:30:1, v:v:v) as the mobile phase and stained with iodine. Tripalmitin (Sigma Aldrich) was used as TAG standard reference. LAS-

Two tomato genes (SlDGAT1-1 Solyc07g0[illegible]
accumulation, SlWSD Solyc01g095960: candidate gene) were cloned, using extracted cDNA, downstream of the constitutive 35S promoter. The different plasmids were generated using ligation independent cloning technique [79] using the binary vector pCV01 [80]. PCR was carried out as followed (primer sequences Suppl. Table B19): five three-step amplification cycles (annealing temperature 45 °C), followed by 30 two-step amplification cycles (annealing/extension temperature 72 °C). The final plasmids were verified by sequencing and transformed into *Agrobacterium tumefaciens* GC3101::pMP90::pSOUP cells.

4-weeks old *A. thaliana* (ecotype Col-0) plants were transformed using floral dip method [81], with a dipping medium containing 5% sucrose and 500 µl/l Silwet L-77. Basta-resistant F1 seedlings were cultivated under short day conditions and leaves were harvested after 5–6 weeks and directly frozen in liquid nitrogen.

3. Results

3.1. Membrane lipid remodeling toward galactolipids and TAG

To investigate the impact of Pi starvation on lipid metabolism, two-weeks old hydroponically grown tomato plants were subjected to Pi starvation (-P) and cultivated in parallel with respective full nutrition (FN) controls. For an initial picture glycerolipids of leaves and roots were analyzed by direct infusion mass spectrometry. Pi starvation caused an increase in the galactolipids DGDG and SQDG in leaves and DGDG and MGDG in root after seven days, while the amounts of nearly all phospholipids were reduced (PA, PC, PE, PG, PI, PS) (Fig. 1). Results indicate phospholipid degradation and galactolipid accumulation, and thus a Pi starvation triggered lipid remodeling of tomato plants.

Analysis further revealed 5.5-fold and 2.5-fold increase of TAG in Pi-starved tomato leaves and roots after seven days, respectively (Fig. 1). So far, TAG accumulation was accepted to be a late response to strong Pi deprivation in plants and algae only induced after 13 days or more [60,82]. Interestingly, *S. lycopersicum* leaves and roots exhibited increased TAG amounts already after three days of Pi starvation (Suppl. Figs. A1 and A2), exhibiting new aspects of lipid remodeling, as TAG accumulated in parallel to galactolipids.

3.2. Galactolipid synthesis mainly from ER-derived precursors

To further investigate the detailed Pi starvation triggered glycerolipid response, molecular species analysis of galactolipids and phospholipids was conducted. DGDG and MGDG molecular species analysis of leaves revealed only minor changes, while root analysis exhibited 2- to 4-fold higher amounts of 34:2, 34:3 and 36:4 galactolipid species (Fig. 2). Since no increase of 34:6 MGDG species (18:3/16:3, assembly only possible via the plastidic pathway, [83]) was detectable in leaves and roots, results suggest assembly of galactolipids from ER-derived precursors. Additionally, the total amount of fatty acids was not significantly affected in tomato leaves (Suppl. Fig. A4), further indicating lipid remodeling rather than galactolipid de novo synthesis.

3.3. Different stress responses of pi-starved leaves and roots in membrane glycerolipids

Detailed galactolipid and phospholipid analysis further revealed major differences of the response of leaves and roots. In leaves, alterations were more drastic in phospholipids, as mainly PC and PE 34:3, methyl ester (FAME) analysis of roots (Suppl. Fig. A5) revealed no changes within the fatty acid composition upon Pi starvation. Hence, data revealed different stress responses of membrane lipids in Pi-starved leaves and roots. In leaves, the amount of galactolipids was only slightly increased upon applied stress conditions, while root tissue revealed major changes in galactolipid levels and composition.

3.4. Alterations in TAG species composition only in Pi-starved tomato leaves

Also TAG composition was further analyzed since TAG levels increased in Pi-starved tomato leaves and roots. The results exhibited that tomato leaves and roots are mainly comprised of 50:X, 52:X and 54:X TAG species (Fig. 3), while TAG species with lower/higher carbon chain length were below the detection limit.

As shown in Fig. 3, almost all TAG lipid species of tomato leaves were affected after seven days of Pi starvation. Leaf TAG exhibited higher levels of polyunsaturated 50:X, 52:X and 54:X, particularly 50:3, 52:5, 52:6 and 54:9. In contrast, tomato roots showed only a marginal increase of 52:5 molecular species and even a decrease of 54:9 molecular species, suggesting lipid composition comparable to full nutrient conditions. Thus, our analysis revealed major differences in the accumulated TAG composition, further indicating different stress responses of lipid metabolism upon Pi starvation conditions in tomato leaves compared to roots.

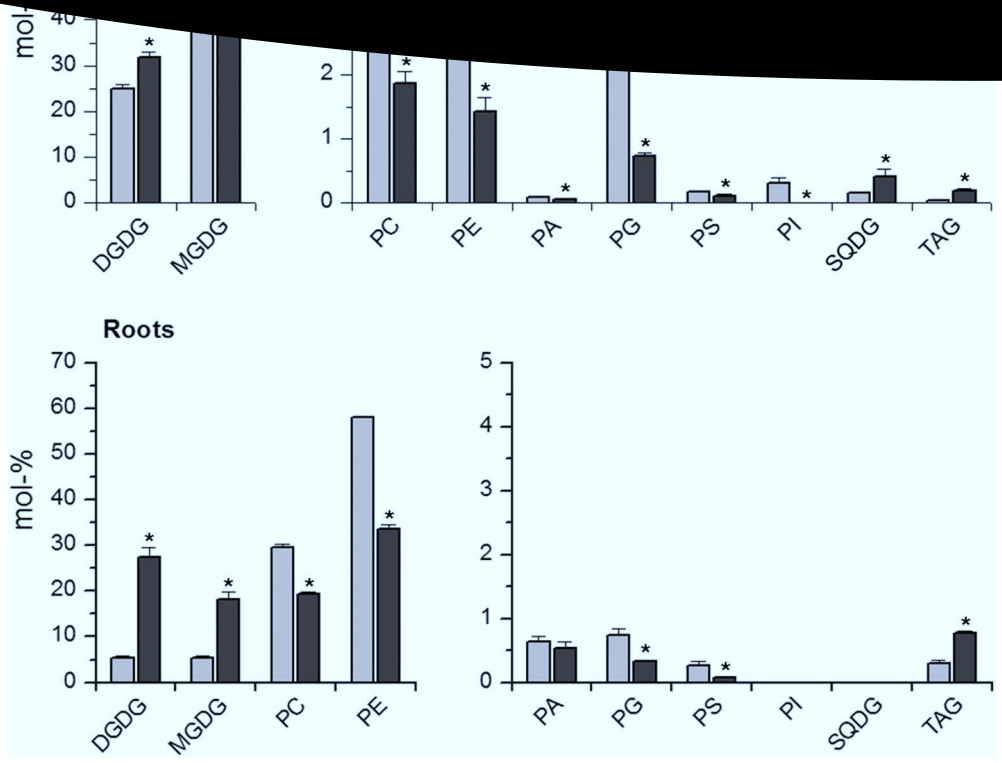
3.5. TAG composition indicates higher desaturation activity on PC in leaf tissue

Higher desaturation level of leaf TAG is most likely due to a higher desaturation level of incorporated 18:X acyl chains, which was confirmed by TAG FAME analysis, exhibiting increased amounts of 18:3 fatty acids (Fig. 4). This higher proportion of 18:3 acyl chains in accumulated TAG in leaves further suggests an increased desaturation activity on acyl chains, which were finally incorporated into TAG. As FAD2 and FAD3 are ER-localized desaturases and primarily act on PC [13], results here suggest higher desaturation activity of FAD2 and FAD3 on PC in Pi-starved leaves, which was confirmed by lipid molecular species analysis (Fig. 2). This PUFA can be further released into the acyl-CoA pool and/or converted into DAG to generate finally TAG [32].

3.6. Phosphate starvation caused changes in transcripts of lipid metabolism

As lipid data revealed phospholipid degradation and conversion into galactolipids and TAG, differential expression analysis of Pi-starved tomato plants was used to further investigate alterations on transcriptional level. To this aim, RNA of leaf and root tissue after one, three and seven days of Pi starvation were sequenced (Suppl. Tables C1 and C2). The samples showed a general agreement between replicates and similar trends across the two different experiments (Suppl. Figs. A6 and A7). In total, after seven days we found increased transcript levels (log2 fold change, logFC > 1; FDR < 0.01) of 2584 (leaves) and 1508 (roots) genes under phosphate starvation, while fewer genes 1766 (leaves) and 674 (roots), showed decreased transcript levels (logFC < -1; FDR < 0.01) (Suppl. Figs. A8 and A9).

To further clarify molecular mechanisms of lipid remodeling upon Pi starvation, *S. lycopersicum* genes putatively involved in lipid metabolism were identified using BLAST+ protein searches against



dard deviation (n = 3 biological replicates, one biological replicate corresponds to two pooled tomato plant samples; results were confirmed in two independent experiments). Asterisk indicates significant changes (P < 0.05).

reference sequences of *A. thaliana* (results Suppl. Table B1). After seven days of Pi starvation over 390 genes, predicted to be involved in lipid metabolism, exhibited higher transcript levels (logFC > 1; FDR < 0.01) in tomato leaves, and approximately 350 genes in tomato roots, while 140 and 160 transcripts (logFC < −1; FDR < 0.01) exhibited lower levels in leaves and roots, respectively (detailed lists of transcriptional changes after all time points see Suppl. Tables B2–B13).

3.7. Lipid remodeling is reflected in transcriptional changes

The rearrangement of membrane lipids upon Pi starvation starts with the dephosphorylation of phospholipids to DAG, which then can be further converted to galactolipids and TAG. After seven days of Pi starvation, the transcripts of phospholipid degrading enzymes were increased (Table 1 and Suppl. Table B5). In particular, phospholipases (NPC1-6 and PLDζ2) and PA phosphatases (PAH1 and PAH2) showed higher transcript levels. Even after three days PLDζ2, PAH1 and PAH2 transcripts were significantly increased (Suppl. Table B11). Furthermore, analysis revealed the induction of galactolipid synthesis genes after three and seven days (MGD2/MGD3, DGD2, UGP3, SQD1, SQD2) (Table 1 and Suppl. Table B10). As MGD2/MGD3 together with DGD2 mainly act on ER-derived precursors [37,84,85], data here further indicate the synthesis of eukaryotic pathway-derived galactolipids and therefore confirm the occurrence of lipid remodeling in Pi-starved tomato plants. Additionally, no transcriptional induction of fatty acid biosynthesis, phospholipid biosynthesis and plastid-pathway derived galactolipid biosynthesis was detectable, further supporting lipid remodeling instead of de novo biosynthesis (7d: Suppl. Tables B2–B4; 1d/3d: Suppl. B8–B11). However, lipid analysis also revealed the accumulation of TAG in Pi-starved leaves and roots, making it worthwhile to further look into TAG assembly metabolism.

3.8. Involvement of DGAT in Pi starvation triggered TAG accumulation

Within the ER, DAG is further acylated to TAG by diacylglycerol acyltransferases DGAT or PDAT, using acyl-CoA or PC as a substrate, respectively [22]. Especially DGAT1 and PDAT1 are responsible for major seed TAG in *A. thaliana* seeds [21]. In the present study, DGAT1-1, DGAT1-2, DGAT3 and PDAT1 transcripts were not affected or even reduced after all measured time points (7d: Table 1 and Suppl. Table B6; 1d/3d: Suppl. Table B12) (for assignment of SlDGATs into DGAT family see Suppl. Fig. A13). Interestingly, our data revealed increased transcript amounts of DGAT2 in tomato leaves after seven days (Table 1), suggesting transcriptional induction upon Pi starvation. In general, in plants that produce oils enriched in unusual fatty acids, like castor bean, tung tree or ironweed, DGAT2 is proposed to be involved in seed oil formation by preferentially incorporating unusual fatty acids into TAG [86–89]. But, since *A. thaliana dgat2* knock-out mutant did not show altered seed TAG formation, authors concluded that AtDGAT2 does not play a substantial role in TAG biosynthesis in developing seeds of *A. thaliana* [21]. Therefore, our results gave a first hint of the possible involvement of SlDGAT2 in the formation of TAG upon Pi starvation in tomato leaves.

However, transcriptional induction of DGAT in leaves and roots seems disproportionate to the increased TAG amount, as especially in roots DGAT transcripts were only slightly affected (Table 1). Furthermore, after three days no increase in DGAT2 transcript amount was detectable in either leaves or roots (Suppl. Table B12), although lipid analysis showed higher TAG levels (Suppl. Figs. A1 and A2). Hence, the activation of DGAT/PDAT proteins and/or other enzymes with diacylglycerol acyltransferase activity possibly determine high amounts of TAG in Pi-starved tissue. Other enzymes with possible diacylglycerol acyltransferase activity have been identified in *A. thaliana*, namely

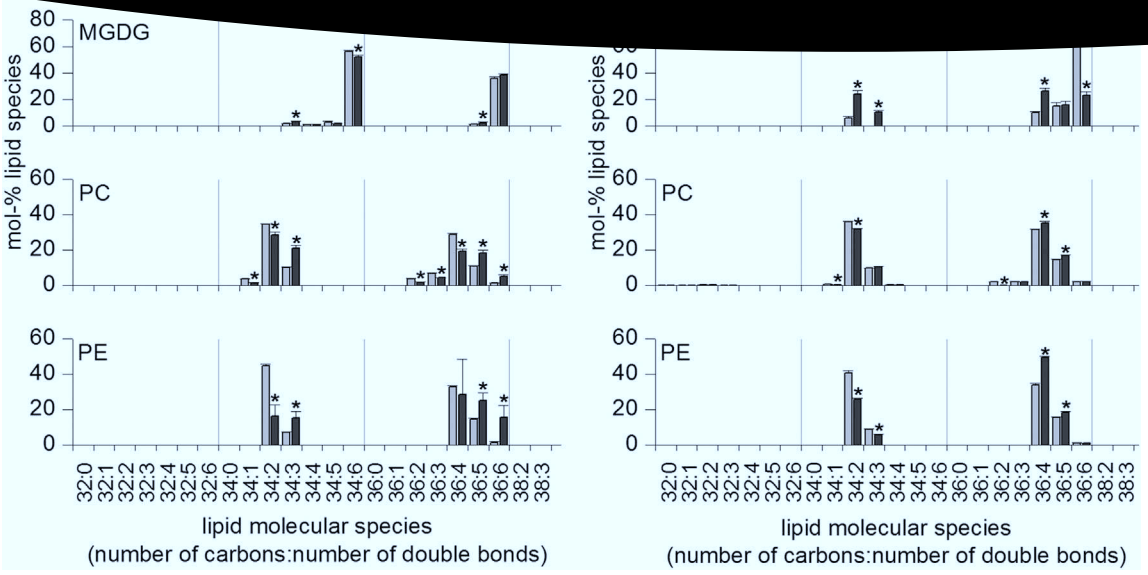


Fig. 2. Changes in glycerolipid composition upon Pi starvation in tomato leaves and roots.
Lipid molecular species of tomato leaves and roots were analyzed by direct infusion tandem mass spectrometry of full nutrient (FN, light gray) and Pi-free (-P, dark gray) grown tomato plants after seven days. The values are the means in mol% (with respect to the analyzed lipids) with the respective standard deviation (n = 3 biological replicates, one biological replicate corresponds to two pooled tomato plant samples; results were confirm in two independent experiments). Asterisk indicates significant changes ($P < 0.05$). Analysis of further lipids (PG, PA, PS, SQDG) see Suppl. Fig. A3.

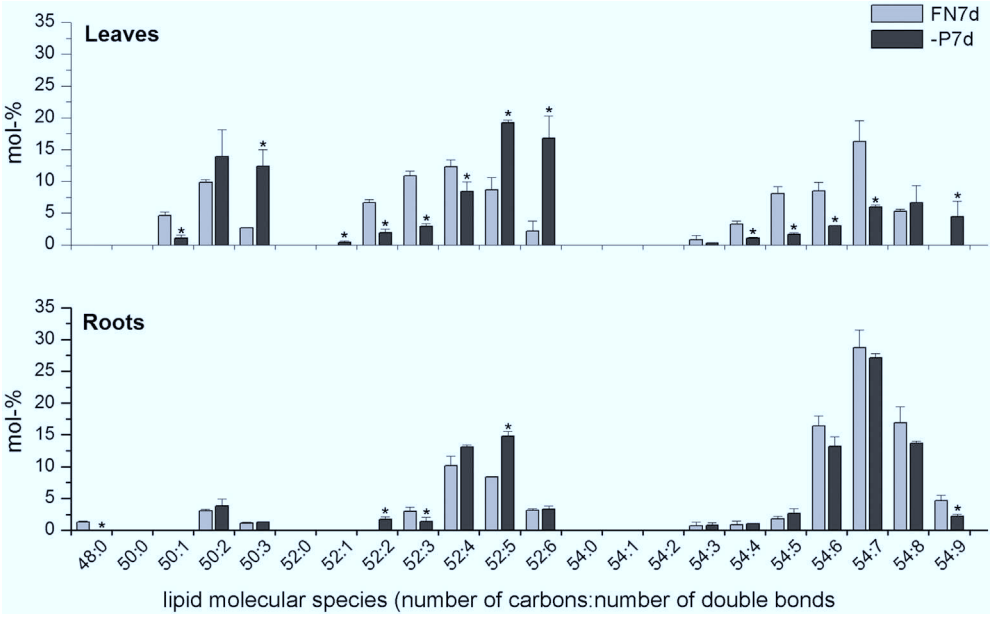


Fig. 3. Changes in TAG composition in Pi-starved tomato leaves and roots.
Lipid molecular species of TAG of tomato leaves and roots were analyzed by direct infusion tandem mass spectrometry of full nutrient (FN, light gray) and Pi-free (-P, dark gray) grown tomato after seven days. The values are the means in mol% (with respect to the analyzed lipids) with the respective standard deviation (n = 3 biological replicates, one biological replicate corresponds to two pooled tomato plant samples; results were confirm in two independent experiments). Asterisk indicates significant changes ($P < 0.05$).

phytyl ester synthase PES1/PES2 [90], wax ester synthase WSD1 [91] and defective in cuticular ridges DCR [92]. Indeed, our data showed increased WSD transcript amounts in tomato leaves after seven days (7d: Suppl. Table B7; 1d/3d: Suppl. Table B13), but an overexpression of SlWSD (Solyc01g095960) could not induce TAG accumulation in *A. thaliana* leaves (Suppl. Fig. A10). This might suggests an involvement of SlWSD in other processes, e.g. wax ester formation, rather than in TAG lipid accumulation upon Pi deprivation.

Hence, the activation of DGAT proteins may explain the discrepancies between missing transcriptional induction of DGATs and increased TAG amounts. To further clarify the possible involvement of DGAT in Pi starvation triggered TAG accumulation, we investigated a radiolabeled activity assay, by measuring the formation of radiolabeled TAG out of [^{14}C] labeled acyl-CoA using microsomal fractions of Pi-starved and full-nutrient leaf and root samples. Results revealed higher capability of seven days Pi-starved microsomal fraction to form

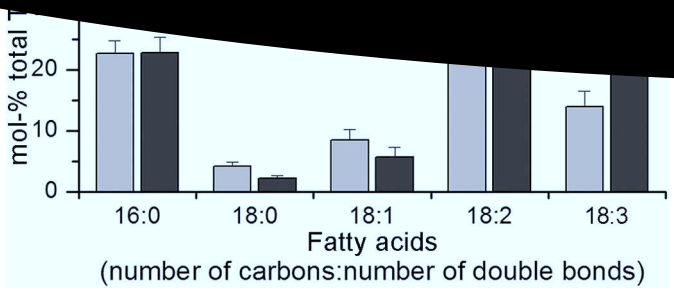


Fig. 4. Pi starvation caused alterations of TAG fatty acid composition in tomato leaves.
Transmethylated fatty acids of TAG, separated by TLC, were analyzed by GC–MS of full nutrient (FN, gray) and Pi-free (-P, dark gray) grown tomato plants of leaves and roots after seven days. The values are the means in mol% (with respect to the analyzed FAME) with the respective standard deviation (n = 3 biological replicates, one biological replicate corresponds to two pooled tomato plant samples). Asterisk indicates significant changes ($P < 0.05$).

radiolabeled TAG out of [^{14}C] acyl-CoA and DAG (Suppl. Figs. A11 and 12). Leaf microsomes already showed a higher radiolabeled TAG proportion after three days of Pi starvation. This result suggests Pi starvation induced TAG biosynthesis at least partly over acyl-CoA as a substrate, catalyzed by DGAT protein in Pi-starved tomato leaves and roots potentially activated e.g. over post-translational activation.

3.9. Comparison of ER-localized fatty acid desaturases in leaves and roots

Since PC and TAG of leaves are highly unsaturated and leaf FAME analysis showed increased amounts of 18:3 acyl chains, FAD2 and FAD3 desaturases might be responsible for higher desaturation level. The tomato genome encodes at least nine FAD2 desaturases and two FAD3 desaturases (assignment see Suppl. Figs. A14, A15 and A16). After seven days of Pi starvation, five FAD2 genes showed high induction in leaves, while just one FAD2 gene showed slight induction in roots (Table 1). Surprisingly, FAD3 exhibited a lower transcript level in leaves and no changes in root tissue after seven days of Pi deprivation. As FAD3 is responsible for the desaturation of 18:2 to 18:3 [29,30], this transcriptional response is in contrast to lipid data, which showed higher amounts of 18:3 especially in TAG FAME analysis of leaf samples (Fig. 4). Nevertheless, data are in line with an observation in seeds of flax whereby LuFAD2 expression increased but not LuFAD3, while only an increase of 18:3 fatty acids was detectable [93]. The authors concluded that the LuFAD2 availability is more limiting for the conversion of 18:1 to 18:3 than LuFAD3 and suggested a regulation of LuFAD2 on transcriptional level and an impact throughout environmental conditions. Therefore, our data may suggest a correlation between FAD2 induction and TAG accumulation, as TAG level and FAD2 transcript amount increased even after three days in Pi-starved tomato leaves (Suppl. Fig. A1 and Table B12).

3.10. Acyl-editing and PC conversion upon Pi starvation

As data suggest a role of SlFAD2 in Pi starvation triggered TAG accumulation, PUFA bound to PC must be released into the acyl-CoA pool and/or PC must be dephosphorylated into DAG, to be available for TAG assembly by DGAT [24,26,32]. In *A. thaliana* seeds, the dephosphorylation to DAG is catalyzed by PDCT (phosphatidylcholine:diacylglycerol cholinephosphotransferase), responsible for 40% of the flux of PUFA from PC over DAG into TAG [25]. Interestingly, in Pi starved

synthase; SQD2: sulfolipid synthase; UGP3: UDP-glucose pyrophosphorylase. qRT-PCR was used to validate RNA-Seq data (Suppl. Table B20). Table illustrates a selection of significantly induced lipid metabolism transcripts. For a detailed listing see Suppl. Tables B2–B13.

Enzyme	Gene ID SI	Leaves 7d (P vs. FN) logFC		Roots 7d (Pvs. FN) logFC		Isoform
DGAT	Solyc07g040740	-0.19		0.17		DGAT1-1
	Solyc12g008970	0.09		0.7	*	DGAT1-2
	Solyc02g068240	1.58	*	0.41		DGAT2
	Solyc12g098850	-5.72	*	-0.48		DGAT3
DGDGS	Solyc01g007100	-0.37		-0.21		DGD1
	Solyc10g081870	0.82		-0.17		
	Solyc10g081900	0.58	*	-0.29		
	Solyc01g094170	1.3	*	0.97	*	DGD2
	Solyc10g017580	3.92	*	2.81	*	
FAD	Solyc04g040120	3.8	*	0.12		FAD2-3
	Solyc04g040130	9.67	*	0.42		FAD2-4
	Solyc12g044950	1.74	*	-0.95	*	FAD2-6
	Solyc12g049030	7.65	*	-0.2		FAD2-7
	Solyc12g100250	5.76	*	1.24	*	FAD2-9
	Solyc06g007130	-1.13	*	-0.19		FAD3-1
	Solyc06g007140	-2.01	*	0.26		FAD3-2
LPCAT	Solyc08g007860	0.23		-0.21		LPLAT1/2
	Solyc08g080340	-0.13		0.03		
	Solyc12g056420	1.6	*	1.15	*	
MGDGS	Solyc08g006640	0.18		0.76	*	MGD1
	Solyc07g007620	3.63	*	5.66	*	MGD2/MGD3
	Solyc12g009820	0.26		0.51	*	
nsPLC	Solyc09g020190	2.67	*	0.65	*	NPC1 - NPC6
PLD	Solyc01g065720	0.21		0.11		PLDζ1
	Solyc01g100020	6.3	*	8.26	*	PLDζ2
PAH	Solyc10g084540	4.3	*	4.45	*	PAH1
	Solyc04g079100	2.96	*	2.57	*	PAH2
pPLA	Solyc04g079210	2.44	*	0.71		pPLA
	Solyc08g006850	4.81	*	-1.65		
SQD	Solyc08g063080	1.17	*	1.93	*	SQD1
	Solyc09g014300	4.78	*	3.45	*	SQD2
	Solyc10g085100	2.36	*	4.52	*	
UGP3	Solyc06g051080	1.45	*	2.73	*	UGP3

tomato leaves and roots, PDCT transcripts are not affected. Also the C/EPT (CDP-choline/CDP-ethanolamine:DAG choline/ethanolaminephosphotransferase) (responsible for the conversion of PC and PE to DAG for the TAG synthesis within the Kennedy Pathway [94,95]) is only slightly affected in tomato leaves and roots after seven days of Pi starvation (Suppl. Tables B2 and B11).

In contrast, our data showed increased transcript levels of acyl-CoA

data suggest PC co

4. Discussion

Plants have evolved a wide range of adaption mechanism to cope with low Pi conditions, including membrane lipid remodeling to utilize phospholipids as internal P source. This process has drawn much attention in the model organism *A. thaliana*, but it is likely that this important process occurs in a wide variety of plant species. Therefore, a combined survey of glycerolipid and differential expression analysis was conducted to investigate the adaption of *S. lycopersicum* leaves and roots to phosphate starvation and to clarify contribution of different enzymes into TAG accumulation mechanisms.

4.1. Confirming lipid remodeling in tomato plants

Our results show that *S. lycopersicum* respond to Pi starvation with a reduction of phospholipids and increased amounts of galactolipids in leaf and root tissue accompanied with higher levels of MGDG (MGD2, MGD3), DGDG (DGD2) and SQDG (UGP3, SQD1, SQD2) synthase transcripts. Thus, data of tomato plants confirm earlier reports of lipid remodeling in *A. thaliana* and oat [42,45,46,52,96], suggesting lipid remodeling toward galactolipids mainly via the MGD2/3–DGD2 interplay [37,50]. In general, nongreen plant organs, like root plastids, lack the large thylakoid membrane system of chloroplast, and therefore have much lower galactolipid contents compared to leaf tissue [97], concurrent with the results of the present study. As in *A. thaliana* and oat plants, increased amounts of galactolipids, especially DGDG, were observed in extraplastidic membranes upon Pi starvation [45,96], the drastic increase of galactolipids in Pi-starved root tissue may also contributes to these findings.

Furthermore, in *A. thaliana* it was shown that NPC4 [39], NPC5 [38,40], PLDζ1 [42], PLDζ2 [38,41] and PAH1/PAH2 [43] are involved in phospholipid degradation under Pi-deficient conditions. Data of the present are in line with these findings and further support transcriptional analysis in *A. thaliana* [38,48,49,51,52,55–59], potato [58] and rice [59], suggesting Pi starvation triggered dephosphorylation of phospholipids via NPC and PLD/PAH pathways.

4.2. New insights into TAG accumulation

Compared to other setups, glycerolipid analysis of hydroponically grown Pi-starved tomato plants revealed an accumulation of TAG already after three days, exhibiting new aspects of the lipid remodeling response. So far, TAG accumulation was assumed to be a late response of strong Pi starvation in plants and algae [60–62,82,98,99] induced after 13 days or more. Compared to nitrogen deprivation, whereby the transcriptional induction of DGAT1 was reported [100,101], phosphate starved tomato plants differ in their transcriptional response. Furthermore, under both stress treatments TAG levels increased, but the TAG compositions were different. Gaude et al. reported higher levels of 16:0 fatty acids and reduced content of 18:3 acyl chains [102], which is in contrast to the present study which showed increased 18:3 fatty acid amounts.

Our data further suggest the involvement of DGAT enzymes in the Pi starvation triggered accumulation of TAG in vegetative tissue, as i) the radiolabeled activity assay revealed higher capability of Pi-starved microsomal fractions to form radiolabeled TAG out of [^{14}C]acyl-CoA, and ii) DGAT2 transcripts were increased in Pi-starved tomato leaves after seven days. So far, DGAT2 was functionally characterized in the yeast mutant strain H1246, indicating diacylglycerol acyltransferase activity [104]. Therefore, our data give a first hint of the involvement of DGATs in Pi starvation induced TAG accumulation in tomato plants, albeit the detailed contribution of different DGAT enzymes needs to be further elucidated.

Higher TAG amounts after three and seven days could not be entirely linked to increased amounts of diacylglycerol acyltransferase transcripts (DGAT or PDAT), raising the question how TAG assembly is proceeded in Pi-starved tissues. Additionally, further reports revealed that TAG per dry weight levels were around 500-fold higher in *A. thaliana* seeds compared to leaves [42,106,107], while DGAT1 transcripts were only 5-fold higher [28], and Schwender et al. reported discrepancies between enzyme activities and transcript levels and highlighted the involvement of e.g. post-translational mechanism in the regulation of central metabolic fluxes [108].

Therefore, we propose posttranslational activation of DGAT or interaction with other proteins may playing additional role in accumulation of TAG, since radiolabeled DGAT-activity assay, using acyl-CoA as substrate, revealed DGAT activity in Pi-starved microsomal membranes. In *A. thaliana*, it was reported that leucine zipper may mediate interactions of DGAT1 subunit with other proteins [23,109,110], and multiple potential phosphorylation sites in DGAT1 were reported [23,110], suggesting a role of posttranslational modifications in the modulation of DGAT activity. Further research should focus on the contribution of the different TAG assembling enzymes in Pi starvation triggered TAG accumulation.

4.3. Composition of precursors determine synthesis of TAG or galactolipids

Our data also revealed major differences in the lipid metabolism response of tomato leaves and roots toward Pi starvation (Fig. 5), which provides insights into how the remodeling directs degraded phospholipids toward galactolipid or TAG formation. The magnitude of alterations within the galactolipids was more drastic in roots, confirming an earlier report in *A. thaliana*, detecting bigger differences in the response of Pi-starved roots [42]. In contrast, a shift toward polyunsaturated TAG and PC was only observed in leaves, indicating different stress responses of tomato leaves and roots upon Pi starvation. Also transcriptional analysis revealed major differences in the response, as the induction of NPC1-5, DGAT2, FAD2 and pPLA was mainly noticeable in leaves after seven days (Fig. 5). Based on the combination of lipidomic and transcriptomic analysis we propose different stress responses in Pi-starved tomato leaves and roots, including dephosphorylation of phospholipids and conversion into TAG and galactolipids. In leaves, upon Pi starvation FAD2 induction leads to the desaturation of incorporated fatty acids in PC, resulting in a polyunsaturated PC pool. The acyl-editing, catalyzed by LPCAT1/2 and pPLA, further causes a polyunsaturated acyl-CoA pool, which triggers the incorporation of PUFA into TAG, leading to a polyunsaturated TAG pool in leaves. Additionally, the transcriptional induction and/or posttranslational activation of DGATs further promote the incorporation of polyunsaturated acyl chains into TAG. In roots, upon Pi starvation phospholipids are likely dephosphorylated mainly by the PLD/PAH pathway. The ER-derived precursors can be further converted into galactolipids. Indeed also in roots, degraded phospholipids are partly converted into TAG, but as ER-localized fatty acid desaturation is not induced, PC and acyl-CoA pools are probably similar to full nutrient conditions, leading to a TAG pool comparable to FN conditions.

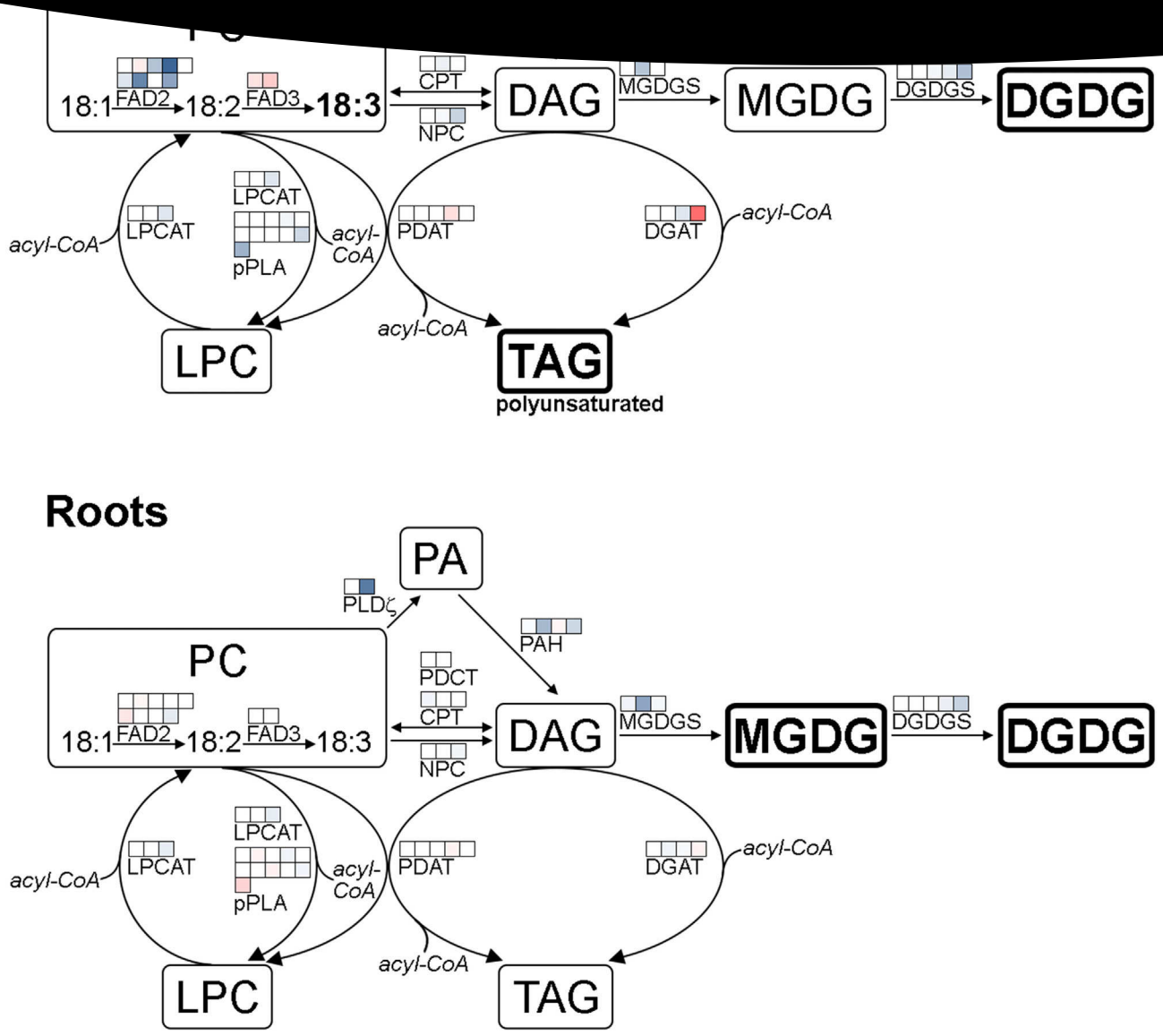


Fig. 5. Different stress responses of tomato leaves and roots upon Pi starvation.
The combined survey of the analysis of glycerolipids and differential expression analysis lead us to propose different stress responses of Pi-starved tomato leaves and roots after seven days. Transcriptional changes (logFC) are indicated by color code next to the enzyme name. Increased amounts of lipid content are marked in bold letters.
Abbr: CPT: CDP-choline:DAG cholinephosphotransferase; DGAT: acyl-CoA:diacylglycerol acyltransferase; DGDGS: DGDG synthase; FAD: fatty acid desaturase; LPCAT: acyl-CoA:lysophosphatidylcholine acyltransferase; MGDGS: MGDG synthase; NPC: non-specific phospholipase C; PLD: phospholipase D; PAH: PA phosphohydrolase; PDAT: phospholipid:diacylglycerol acyltransferase; PDCT: phosphatidylcholine:diacylglycerol cholinephosphotransferase; pPLA: patatin-related phospholipase A.

This occurrence of separate substrate pools and their different composition possibly explains the modulation toward galactolipids or TAG. Bates et al. (2009) already concluded, from their quantitative analysis of acyl group and glycerol backbone flux analysis using [^{14}C] acetate and [^{14}C]glycerol in developing soybean embryos, the existence of separate pools of DAG for TAG and PC biosynthesis. As the concept of different endomembrane domains is well known [111] it was suggested that each DAG pool represents a separate ER domain [25]. Also Vogel et al. (1996) postulated distinctly separated DAG pools as an explanation for the incorporation of unusual fatty acids into TAG and their exclusion from membrane lipids, rather than on the basis of DGAT substrate specificities [112].

Several additional studies support the hypothesis that the availability and composition of the acyl-CoA pool and ER-derived precursors determine the mediation of degraded phospholipids toward triacylglycerol or galactolipid synthesis. Kim et al. suggested that the overexpression of the ER-localized ABCA9 (ATP-binding cassette) transporter leads to an increased acyl-CoA pool within the ER, promoting the

seeds, but no alter
an impact of acyl-CoA amounts on seed TAG
further study it was shown that in oil palm, which mainly accumulates oil within the mesocarp, only transcriptional values of FAD2 and DGAT2 were increased compared to date palm, which almost exclusively contains sugar, supporting our hypothesis that the induction of FAD2 and DGAT2 correlates with higher TAG amounts [115].

Nevertheless, a substrate specificity of DGAT cannot be completely ruled out. The dgat1 mutant of *A. thaliana* showed a slight increase in total saturated TAG [18], which may indicate a slight preference for saturated substrates. Furthermore, it was reported that DGAT2 preferentially incorporates unusual fatty acids into seed TAG in tung tree [86], castor bean [87,88] and ironweed [89]. Also the complementation of the yeast mutant strain H1246 with either AtDGAT1 or AtDGAT2 led to different fatty acid compositions [104].

However, our data clearly showed different stress responses of tomato leaves and roots and indicate the involvement of SlFAD2 and SlDGAT in Pi starvation triggered TAG accumulation, which enhances our understanding of the Pi starvation response of lipid metabolism.

5. Conclusion

In conclusion, we observed lipid remodeling toward galactolipids in Pi starved tomato plants, combined with an early increase of TAG, at least partly catalyzed by DGAT enzymes. In addition, data revealed remarkable differences of leaves and roots in TAG and galactolipid content/composition, as well as transcriptional induction of phospholipid degradation, ER-localized fatty acid desaturation and triacylglycerol assembly. Therefore, our data support a model postulating that the availability and composition of the acyl-CoA pool and ER-derived precursors possibly determine the modulation of dephosphorylated phospholipids toward TAG and galactolipid biosynthesis in Pi-starved tomato plants. In leaves, TAG biosynthesis is possibly driven by a highly unsaturated acyl-CoA and PC pool, due to fatty acid desaturase activity and acyl-editing, while in roots, Pi starvation resulted in an increased biosynthesis of galactolipids, which is supposed to be mediated by step wise degradation of phospholipids to PA and finally DAG.

Transparency document

The Transparency document associated this article can be found, in online version.

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CRediT authorship contribution statement

Julia Pfaff: Conceptualization, Methodology, Investigation, Writing - original draft, Visualization, Funding acquisition. **Alisandra K. Denton:** Formal analysis, Writing - review & editing. **Björn Usadel:** Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition. **Christian Pfaff:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision.

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