Article title: Inducible expression of DEFECTIVE IN ANTHER DEHISCENCE 1 enhances triacylglycerol accumulation and lipid droplet formation in vegetative tissues

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SUMMARY

Bioengineering efforts to increase oil in non-storage vegetative tissues, which constitute the majority of plant biomass, are promising sustainable sources of renewable fuels and feedstocks. While plants typically do not accumulate significant amounts of triacylglycerol (TAG) in vegetative tissues, we report here that the expression of a plastid-localized phospholipase A1 protein, DEFECTIVE IN ANTHER DEHISCENCE1 (DAD1), led to a substantial increase in leaf TAG in Arabidopsis. Using an inducible system to control DAD1 expression circumvented growth penalties associated with overexpressing DAD1 and resulted in a rapid burst of TAG within several hours. The increase of TAG was accompanied by the formation of oil bodies in the leaves, petioles, and stems, but not in the roots. Lipid analysis indicated that the increase in TAG was negatively correlated with plastidial galactolipid concentration. The fatty acid (FA) composition of TAG predominantly consisted of 18:3. Expression of DAD1 in the *fad3fad7fad8* mutant, devoid of 18:3, resulted in comparable TAG accumulation with 18:2 as the major FA constituent, reflecting the flexible *in vivo* substrate use of DAD1. The transient expression of either Arabidopsis DAD1 or *Nicotiana benthamiana* DAD1 (NbDAD1) in *N. benthamiana* leaves stimulated the accumulation of TAG. Similarly, transgenic soybeans expressing Arabidopsis DAD1 exhibited an accumulation of TAG in the leaves, showcasing the biotechnological potential of this technology. In summary, inducible expression of a plastidial lipase resulted in enhanced oil production in vegetative tissues, extending our understanding of lipid remodeling mediated by DAD1 and offering a valuable tool for metabolic engineering.

Keywords: leaf oil, biofuels, storage lipid, phospholipase, PLA1, DAD1, triacylglycerol

INTRODUCTION

Escalating concerns about environmental sustainability and the depletion of fossil fuels have resulted in a renewed interest in vegetable oil-based alternatives to petroleum fuel (Ortiz et al., 2020; Singh et al., 2021; Vanhercke et al., 2019). One approach is to bolster oil production in

vegetative tissues of plants; however, oils are not typically found at high levels in leaves and stems (Durrett et al., 2008; Vanhercke et al., 2014; Xu and Shanklin, 2016). In addition to increasing the potential for overall higher oil production, the use of vegetative tissues to store oil can diversify the range of plant species functioning as bioenergy crops, alleviating concerns about competition for resources dedicated to food production (Dyer et al., 2008). The increased energy density and improved quality, including a healthier fatty acid (FA) profile, in vegetative tissues that are desirable for fodder and silage crops can directly impact both animal health and the quality of meat and dairy products (Knothe, 2010; Winichayakul et al., 2013).

To achieve this goal, plant metabolic engineering efforts have focused on manipulating genes involved in the carbon partitioning and production of lipids. Examples include manipulating single genes or combinations of multiple genes encoding transcription factors (Baud et al., 2007; Kim et al., 2013; Sanjaya et al., 2011; Zhai et al., 2017), enzymes that partition carbon to pyruvate for FAs (Morley et al., 2023), proteins catalyzing the committed steps for FA biosynthesis (Liu et al., 2019; Salie et al., 2016; Wang et al., 2022; Ye et al., 2020), proteins involved in triacylglycerol (TAG) assembly (Cao et al., 2023; Fan et al., 2013b; Luo et al., 2022; Singer et al., 2016; Vanhercke et al., 2014; Yurchenko et al., 2018), packaging into lipid droplets (LDs) (Cai et al., 2015; Cai et al., 2017; Gidda et al., 2013; Ischebeck et al., 2020; Pyc et al., 2021), as reviewed elsewhere (Metzger and Bornscheuer, 2006; Ortiz et al., 2020; Singh et al., 2021; Vanhercke et al., 2019; Xu and Shanklin, 2016). Additionally, carbon from starch was redirected to oil biosynthesis or TAG lipases or peroxisomal enzymes were targeted to block lipid turnover, to further enhance oil accumulation in vegetative tissues (Azeez et al., 2022; Aznar-Moreno et al., 2022; Eastmond, 2006; Kelly et al., 2013; Sanjaya et al., 2011; Slocombe et al., 2009; Xu et al., 2019).

Despite the success thus far, the capacity for much greater levels of storage oil may be possible as evidenced by changes in TAG concentration during stresses, such as elevated temperature, freezing, nitrogen deprivation, drought, exposure to ozone, wounding, or pathogen infection (Coulon et al., 2024; El Hafid et al., 1998; Lewandowska et al., 2023; Lippold et al., 2012; Moellering et al., 2010; Mueller et al., 2015; Narayanan et al., 2016; Pant et al., 2015; Sakaki et al., 1990; Schieferle et al., 2021; Shimada and Hara-Nishimura, 2015; Vu et al., 2015; Yang and

Benning, 2018; Yurchenko et al., 2018). Additionally, senescence can promote TAG accumulation (Coulon et al., 2024; Kaup et al., 2002; Lu et al., 2020; Troncoso-Ponce et al., 2013; Watanabe et al., 2013), highlighting the plasticity of lipid production in vegetative cells. However, these effects are transient, as TAG molecules can be quickly removed by β-oxidation or converted into other metabolites (James et al., 2010; Koo et al., 2005; Theodoulou and Eastmond, 2012; Tjellstrom et al., 2015). Further, vegetative tissues with significant storage oil accumulation can exhibit reduced plant growth (Kelly et al., 2013; Kim et al., 2013; Sanjaya et al., 2011; Xu et al., 2005; Zhai et al., 2021), suggesting the oil production may be a costly process. In some instances, particularly in tobacco, the plants are comparable or only slightly smaller in size (Chu et al., 2022; Vanhercke et al., 2017; Zhou et al., 2020); thus, the underlying physiology that results in altered plant size when lipid levels increase remains enigmatic. Perhaps there is selection pressure against storing high-energy nutrients in vegetative tissues, considering the heightened vulnerability to insect pests in plants with elevated oil content (Sanjaya et al., 2013; Yurchenko et al., 2018). Alternatively, the costs associated with intensive lipid production compared to other forms of biomass may be significant enough to hinder plant growth.

The fatty acyl building blocks used for TAG assembly in non-storage organs during stress responses are likely derived from membrane lipids hydrolyzed by lipases (Higashi et al., 2018; Lippold et al., 2012; Pant et al., 2015; Sakaki et al., 1990; Shimada and Hara-Nishimura, 2015; Troncoso-Ponce et al., 2013; Vu et al., 2015; Wang et al., 2018; Yu et al., 2021) or through reverse reactions of acyltransferases (Tjellstrom et al., 2015). Phospholipases, a major class of lipid hydrolases, can cleave fatty acyl groups from various glycerol lipid substrates that constitute most cell membranes. Several class A phospholipases (PLAs) have been implicated in membrane remodeling under various stress conditions (reviewed in (Ali et al., 2022; Chen et al., 2013; Kelly and Feussner, 2016; Laxalt and Munnik, 2002; Wang et al., 2012; Yu et al., 2021), and some have been shown to be involved in TAG accumulation (Wang et al., 2017). PLASTID LIPASE1 (PLIP1) in Arabidopsis hydrolyzes fatty acyl group from phosphatidylglycerol (PG) from chloroplast thylakoids and contributes to oil accumulation during seed development (Aulakh and Durrett, 2019; Wang et al., 2017) and galactolipases (e.g., PGD1) can release fatty acyl groups from monogalactosyldiacylglycerol (MGDG) for TAG synthesis as noted for nitrogen starved green algae (Li et al., 2012). A portion of FAs hydrolyzed from membrane

lipids is converted to oxylipins including jasmonates (Kallenbach et al., 2010; Kimberlin et al., 2022), which are an important class of phytohormones that protect plants against diverse abiotic and biotic stresses (Howe et al., 2018; Koo, 2018). In Arabidopsis, seven PLA1s with predicted plastid transit peptides have been identified: DEFECTIVE IN ANTHER DEHISCENCE1 (AtDAD1; At2g44810), DONGLE (DGL; At1g05800), PLA1-Iα2 (At2g31690), PLA1-Iβ2 (At4g16820), PLA1-Iγ1 (At1g06800), PLA1-Iγ2 (At2g30550), and PLA1-Iγ3 (At1g51440) (Rudus et al., 2014; Ryu, 2004). Several of these plastidial PLA1s including AtDAD1, contribute redundantly to oxylipin biosynthesis in Arabidopsis (Ellinger et al., 2010; Hyun et al., 2008; Ishiguro et al., 2001; Morin et al., 2023). Additionally, two abscisic acid (ABA)-responsive paralogs of the above-mentioned PLIP1 have been reported to contribute to oxylipin biosynthesis (Wang et al., 2018). The HEAT INDUCIBLE LIPASE1 when knocked out reduced heat-induced TAG accumulation in Arabidopsis leaves at the expense of MGDG (Higashi et al., 2018). Both PLIP1 and HIL1 are distinct from the seven DAD1-like plastidial PLA1s. While involvement of lipid hydrolysis in TAG biosynthesis in leaves is evident, there have been no concerted efforts to alter leaf oil by manipulating plastidial PLA1s.

In this study, we report oil accumulation in vegetative tissues by expression of a plastidial PLA1 (Figure 1a). A chemical-inducible expression system was employed to overcome the growth inhibitory complications associated with constitutively expressing PLA1s (Ishiguro et al., 2001; Kimberlin et al., 2022; Wang et al., 2017). The resulting plants exhibited conditional accumulation of a substantial amount of TAG in the vegetative tissues in response to the exogenous application of a chemical inducer. The TAG predominantly consisted of 18:3, accompanied by a concomitant decrease in MGDG and digalactosyldiacylglycerol (DGDG). Heterologous expression in *Nicotiana benthamiana* and *Glycine max* resulted in similar increases in leaf TAGs. We discuss how this unique approach can be leveraged and integrated with existing strategies to advance efforts in developing biofuel crops.

RESULTS

Transient expression of AtDAD1 under an inducible promoter leads to TAG accumulation in leaves

We previously reported on the generation of transgenic lines (*Pdex:AtDAD1-Myc*) carrying a dexamethasone (dex)-inducible construct expressing AtDAD1-*Myc* and characterization of jasmonic acid (JA) metabolism (Holtsclaw et al., 2024; Kimberlin et al., 2022). Based on those studies, we hypothesized that the ectopic expression of AtDAD1 would hydrolyze membrane glycerolipids within plastids, liberating free fatty acids (FAs) like α-LA, which would then be metabolized via pathways beyond the JA biosynthetic route to produce TAG (Figure 1a).

Consistent with previous findings, AtDAD1-*Myc* did not express in *Pdex:AtDAD1-Myc* plants in the absence of exogenously applied dex, a compound not biosynthesized in planta. This was confirmed through mRNA transcript analysis and protein immunoblot using commercial *Myc* antibodies to detect recombinant AtDAD1-*Myc* (Figure S1a and b). Upon an application of dex, there was distinct induction of both *AtDAD1-Myc* transcripts and proteins within 12 h (Figure S1a and b). Earlier induction of both transcripts and proteins was reported within 4 h (Kimberlin et al., 2022). Lipid analysis was conducted on leaf samples collected from *Pdex:AtDAD1-Myc* plants at 0, 4, 8 and 12 h after dex treatment, and those were compared with lipids extracted from control WT plants similarly treated with dex for 12 h. TLC plate separation of the total lipid extract, utilizing a hexane/diethyl ether/acetic acid (80/20/1, v/v/v) solvent system, revealed time-dependent appearance of TAG bands in the dex-treated *Pdex:AtDAD1-Myc* samples (Figure S1c). No clear TAG band could be detected from the *Pdex:AtDAD1-Myc* samples prior to the dex treatment (0 h) similar to the WT control.

Quantitative analysis by GC-FID of leaf TAGs ranged from 300 μg/gFW at 4 h to 400-500 μg/gFW at 8 h and 12 h post dex treatment (Figure 1b). No statistically significant changes were observed in total leaf lipid contents (converted to FA methyl ester (FAME)) of dex-induced *Pdex:AtDAD1-Myc* compared to WT over time (Figure 1c), indicating no net gain or loss in overall acyl lipid quantity. In order to verify that the TAG increase in the leaves of *Pdex:AtDAD1-Myc* was dependent on the activity of AtDAD1, a transgenic line expressing a mutated variant of AtDAD1 was examined (*Pdex:AtDAD1^{mut}-Myc*) (Figure 1d and e). The mutant construct housed in this line had the AtDAD1 with highly conserved GXSXG motif that included catalytic Ser^{295} residue replaced with five alanine residues controlled by the same dex-inducible expression system (Kimberlin et al., 2022). Lipid analysis of *Pdex:AtDAD1^{mut}-Myc* exhibited no noticeable increase in TAG following dex treatment (Figure 1d and e). This

indicated that the rise of TAG within the *Pdex:AtDAD1-Myc* lines is contingent upon the catalytic activity of the functional AtDAD1 enzyme.

Lipid droplets form in the leaves and stems of dex-induced Pdex:AtDAD1-Myc

Where does the accumulated TAGs localize within the cellular context? Previous studies have shown the leaf tissue's potential to amass lipid droplets (LD) (Bouchnak et al., 2023; Pyc et al., 2017a), typically found in oil-rich tissues such as seeds. When stained with BODIPY and examined using a laser scanning confocal microscope, punctate structures that are typical for LDs (green in the image), much smaller than chloroplasts (red), emerged in dex-treated (8 h) *Pdex:AtDAD1-Myc* leaves (Figure 2a). A secondary oil staining approach with Nile Red further confirmed the oil-filled nature of these organelles (Figure 2a). Notably, LDs were absent in mock-treated (0.01% Triton X-100 in water) *Pdex:AtDAD1-Myc* lines or dex-treated WT leaves (Figure 2a). As time progressed (12 h post dex treatment) the presence of LDs intensified in the *Pdex:AtDAD1-Myc* lines (Figure 2a). To investigate LD formation in tissues other than leaves, lipid staining was carried out in petiole, stem, and root tissues. Similar to the leaf tissue, LDs formed in dex-treated (12 h) petiole and stem tissues of *Pdex:AtDAD1-Myc*, in contrast to WT, which did not display any perceivable LD stain (Figure 2b). Interestingly, LDs failed to form in the roots (Figure 2b), even though the control root tissues that had been fed with exogenous α-LA, displayed lipid stains (Figure 2b), consistent with the root's capacity to accumulate LDs (Kelly et al., 2013; Pyc et al., 2017a; Pyc et al., 2017b). These findings align with previously reported transgenic lines with increased oil content showing LD formation in vegetative tissues (Cai et al., 2017; Chu et al., 2022; Gidda et al., 2016; Winichayakul et al., 2013). The reasons for the limited accumulation of TAG in the roots of *Pdex:AtDAD1-Myc* are unclear, but it could likely be due to the lack of extensive internal membrane system (thylakoids) in root plastids (Xue et al., 1997).

Pdex:AtDAD1-Myc plants can be induced to accumulate leaf TAGs at various developmental stages

Undesirable agronomic traits like stunted growth, diminished yield, or heightened insect herbivory (Sanjaya et al., 2011; Yurchenko et al., 2018; Zhai et al., 2021), may be avoided if oil accumulation can be transiently induced at specific, desired times. The dex-inducible system is

under tight regulation, resulting in minimal, if any, leakage of transgene expression (Figure S1) (Koo et al., 2009). Consequently, *Pdex:AtDAD1-Myc* plants prior to dex induction exhibited normal growth, similar to WT plants (Figure 3a and b). Meanwhile, both *AtDAD1-Myc* transcripts and proteins were induced to comparable levels by dex at various developmental stages (Figure 3c and d). These observations underscore the effective functionality of the inducible vector system across most developmental stages of rosette leaves. Qualitative assessment of oil accumulation in these samples revealed that, except for 8-d-old plants, TAG was detectable at similar levels in samples from the five remaining developmental stages (11-26 d) (Figure 3e). Although the sample from 8-d-old plant in the TLC image (Figure 3e) appears to be slightly underloaded, the absence of the TAG band was evident. Given that this sample exhibited equivalent levels of *AtDAD1-Myc* transcripts and proteins as other plant age samples (Figures 3c and d), the lack of TAG was surprising. However, this observation aligns with a previous report where the induced AtDAD1 proteins were also unable to trigger JA accumulation early in development (< 10-d-old) (Kimberlin et al., 2022).

Leaf TAG formation primarily occurs at the expense of MGDG and DGDG

Although classified as a phospholipase, some PLA1s exhibit additional substrate specificity for galactolipids (Hyun et al., 2008; Ishiguro et al., 2001; Kallenbach et al., 2010). Considering AtDAD1's localization in the plastids (Ishiguro et al., 2001; Padham et al., 2007) and the prevalence of MGDG and DGDG (constituting > 60% of leaf lipids and > 75% of plastid lipids) (Browse and Somerville, 1994; Welti et al., 2002), these galactolipids are expected to be the primary substrates for AtDAD1. In line with this prediction, a noticeable reduction in MGDG and DGDG levels was observed upon dex treatment in *Pdex:AtDAD1-Myc* leaves (Figure 4a). The decline in MGDG was particularly pronounced 12 h post dex-induction, as indicated by GC analysis (Figure 4b). However, the reduction in individual MGDG lipid species was more evident even at earlier time points (4 h and 8 h), in a separate set of samples analyzed by lipidomics (Figure 4d). Statistically significant decreases ($P < 0.05$) in DGDG were also observed at 4 and 8 h post dex treatment (Figure 4e). The combined levels of MGDG and DGDG were reduced by 200-250 μg/gFW compared to either WT or untreated *Pdex:AtDAD1-Myc* plants.

Among the MGDG species, three major variants—MGDG (34:6), MGDG (36:6), and MGDG (34:5)—exhibited substantial decreases in their levels over time following dex treatment (Figure 4d) (Table S1). These lipid species all contained 18:3 FA (although MGDG (34:5) likely contained some 18:2-16:3)). Similarly, levels of all major species of DGDG lipids—DGDG (36:6), DGDG (34:3), and DGDG (34:6)—with 18:3 fatty acyl groups were reduced following dex treatment. In contrast, changes in phospholipids such as PC, PG, PE, PS, PI, and PA were more subtle, with no overt shifts detected in overall levels (Figure S2). Nonetheless, individual PC species exhibited more complex patterns: some species (PC (36:6), PC (34:3), PC (34:4)) increased, while others (PC (36:4), PC (36:3), PC (36:2), PC (34:2), PC (34:1)) decreased, and certain species remained unchanged (PC (36:5)) (Figure S3a). Additionally, there was an increase in LysoPC containing 18:3 and 18:2 FAs (Figure S3b). The fluctuations in PC could reflect the transition of FAs that have been released from the plastid through PC intermediates as part of the PC-acyl editing cycle (Allen, 2016; Bates, 2016; Tjellström et al., 2012) on their paths to being incorporated into TAG (Figure 1a). Notably, a reduction in one PG species (34:4) was also observed (Figure S3c). The change in PG suggests that the action of AtDAD1 may not be solely limited to MGDG and DGDG.

Leaf TAG in Pdex:AtDAD1-Myc mainly consists of unsaturated 18-carbon FAs

Next, we analyzed the FA composition of leaf TAG in *Pdex:AtDAD1-Myc*. WT and uninduced *Pdex:AtDAD1-Myc* plants accumulated less than 10 μg/gFW TAG (Figure 5a), and the small amount they did accumulate primarily consisted of saturated 16 and 18-carbon FAs. However, 50-60% of TAGs from dex-induced *Pdex:AtDAD1-Myc* consisted of 18:3 (Figure 5a and b). The remaining 50% contained 18:2, 16:0, 18:0, 16:3, and 18:1 in decreasing order of relative abundance. This FA profile remained consistent over time, except for 16:3 and 18:1, which showed opposite trends of decreasing and increasing, with their relative abundance ultimately reversing by 12 h post-dex treatment (Figure 5a). These FA profiles stand in stark contrast to Arabidopsis seed oil, which mainly consists of 18:2 (30%), followed by 20:1 and 18:3, each accounting for about 20% (Browse and Somerville, 1994; Li et al., 2006). The predominance of 18:3 in *Pdex:AtDAD1-Myc* TAG reflects that in MGDG and DGDG (Figure 5b). The main difference between TAG and galactolipid FA profile lies in the relatively high 18:2 content (20-25%) in TAG, compared to larger 16:3 content (25-30%) in MGDG (Figure 5b). The exclusion

of 16:3 in leaf TAG is likely due to the predominant occurrence of 16:3 in the sn-2 position of MGDG (Miquel and Browse, 1992) and AtDAD1 being a PLA1 enzyme.

Lipidomic analysis of *Pdex:AtDAD1-Myc* leaf tissues revealed acyl species in TAG (Figure 5c) (Table S1). Consistent with prior results, very little TAG was present in WT or uninduced (0 h) *Pdex:AtDAD-Myc* plants; however, leaf samples collected after 4 h and 8 h of dex treatment contained several TAG species (Figure 5c). The two most abundant species were TAG (54:8) and TAG (54:9), consisting of TAG (18:2_18:3_18:3) and TAG (18:3_18:3_18:3), respectively. The next four most abundant TAG species all contained 18:3. The unsaturation index also increased in TAG and the extraplastidial phospholipids PC and PI (and PA), while it decreased in the plastidial glycerolipids MGDG, DGDG and PG (Figure S4). These FA profiles in TAGs and double bond contents in lipids reflect the expected outcomes of plastidial PLA1 hydrolyzing glycerolipids, particularly, MGDG and DGDG, releasing unsaturated 18-carbon FAs, ultimately culminating in highly unsaturated TAG molecules within the leaves.

Pdex:AtDAD1-Myc can promote TAG accumulation in fad3fad7fad8 mutant

The sterility phenotype of *dad1* mutant plants, attributed to the absence of the plant hormone JA, is caused by the defect in the release of 18:3, serving as the precursor for JA biosynthesis (Ishiguro et al., 2001). This coupled with the FA composition analysis results, showing the predominance of 18:3 in the leaf TAG of *Pdex:AtDAD1-Myc* (Figure 5), suggest that the main substrate of AtDAD1 enzyme is 18:3-containing galactolipids. To further probe the *in vivo* specificity of the AtDAD1 enzyme for 18:3-containing lipids, we introduced *Pdex:AtDAD1-Myc* into the FA desaturase triple mutant, *fad3fad7fad8*, which lacks 18:3 (McConn and Browse, 1996).

Similar to the results observed in the WT background, dex-treated *Pdex:AtDAD1-Myc* in *fad3fad7fad8* background exhibited TAG accumulation in leaves (Figure S5). Notably, there was no significant difference in the total leaf TAG levels between these two genetic backgrounds (Figure S5a). However, lipidomic analysis revealed a major shift in acyl compositions within TAGs. TAGs containing 18:3 were almost completely absent, while TAGs containing 18:2, such as TAG (18:2_18:2_18:2) and TAG (18:2_18:2_18:1), accumulated in *Pdex:AtDAD1-Myc / fad3fad7fad8* (Figure S5b). Additionally, there was a higher abundance of TAGs containing 18:1 and 18:0 in the *fad3fad7fad8* background compared to the WT background (Figure S5b). These

findings indicate that even in the absence of 18:3, the AtDAD1 enzyme retains an ability to hydrolyze 18 carbon FA with varying levels of saturation from glycerolipids in leaves.

Wounding enhances TAG accumulation in Pdex:AtDAD1-Myc

Our previous research on the role of AtDAD1 in JA biosynthesis revealed that co-treatment with wounding and dex significantly enhances JA production in *Pdex:AtDAD1-Myc* plants compared to either of the single treatments. The evidence suggests the activation of AtDAD1 activity through a post-transcriptional mechanism triggered by wounding, resulting in increased JA production (Holtsclaw et al., 2024; Kimberlin et al., 2022). Given that AtDAD1 generates precursors for both JA and TAG, we hypothesized that wounding might also enhance TAG accumulation in dex-induced *Pdex:AtDAD1-Myc* plants.

Leaves that had been pretreated with dex for 8 h were subsequently wounded for 8 h. Wounding alone did not cause a significant increase in TAG compared to the no-wounding control (Figure S6) under our condition, somewhat different from earlier reports that found increases (Lewandowska et al., 2023; Vu et al., 2014). Treatments with dex increased TAG to about 500 μg/gFW (Figure S6) in *Pdex:AtDAD1-Myc* as observed earlier (Figure 1). However, co-treatment with both dex and wounding increased TAG levels to a higher level (750 μg/gFW). This is consistent with observations made for JA biosynthesis (Kimberlin et al., 2022), although the magnitude of TAG increase by the 'dex+wound' co-treatment was smaller than that was observed for JA.

Transient expression of AtDAD1 and NbDAD1 promoted TAG accumulation in N. benthamiana leaves

With the long-term goal of developing crops with high biomass oil content, we conducted experiments to assess whether *Pdex:AtDAD1-Myc* can induce TAG accumulation in other plant species. We selected *N. benthamiana* as a suitable laboratory system for quick testing and evaluation of the effects of transient gene expression. Previous successes in producing oil in tobacco leaves have been reported (Cai et al., 2017; Chu et al., 2022; Gidda et al., 2016; Vanhercke et al., 2017; Vanhercke et al., 2014; Zhou et al., 2020). In addition to the *AtDAD1* gene from Arabidopsis, we also tested a homolog to *AtDAD1* found in *N. benthamiana*. A BLAST query of Sol Genomics Network (genome release, v1.0.1) using the full-length

Arabidopsis AtDAD1 amino acid sequence identified four sequences with sequence identities above 60% (Niben101Scf04104g03001.1, Niben101Scf05795g00005.1, Niben101Scf02400g02012.1, Niben101Scf02386g01005.1) (Holtsclaw et al., 2024). From these, Niben101Scf02386g01005.1 was selected, which was predicted to be localized in the plastids by TargetP2.0 (Almagro Armenteros et al., 2019), and designated it as NbDAD1.

The full-length *NbDAD1* gene was cloned into the dex-inducible vector system (*Pdex:NbDAD1*), and Agrobacteria carrying *Pdex:NbDAD1* were syringe-infiltrated into 4-week-old *N. benthamiana* leaves. Agrobacteria carrying an empty vector or the *Pdex:AtDAD1-Myc* construct were also infiltrated. After 2 days, the infiltrated leaves were sprayed with a 30 μM dex solution and incubated for another 6 h before collecting tissue samples for RNA and oil analyses. The reverse transcriptase (RT)-PCR analysis showed clear induction of *NbDAD1* and *AtDAD1* transcripts in leaves infiltrated with either constructs, compared to *EV* infiltrated leaves (Figure 6a). *AtDAD1* primers cross-reacted with *NbDAD1* in *Pdex:NbDAD1* infiltrated samples. This is presumably due to the high expression level of *NbDAD1* in those samples because the non-specific amplification was not observed in the *EV* control (Figure 6a).

The TLC stain of the oil samples revealed a clear induction of TAG bands in leaves infiltrated with either *Pdex:AtDAD1-Myc* or *Pdex:NbDAD1*, compared to *EV* control (Figure 6b). These results were reproducible in four biological repetitions of each construct. The leaf TAG amounts of 200-450 μg/gFW (Figure 6c) in the leaves expressing either constructs were comparable to those observed in Arabidopsis *Pdex:DAD1-Myc* leaves (Figure 1b). Similar to Arabidopsis (Figure 4), a visible reduction of galactolipids (especially DGDG) was observed in *N. benthamiana* leaves expressing *Pdex:AtDAD1-Myc* compared with *EV*-infiltrated leaves (Figure S7).

Development of transgenic soybeans with increased biomass oil

After obtaining positive results supporting the potential to use inducible PLA1s for engineering biomass oil lines in Arabidopsis and *N. benthamiana*, we transformed soybean (*Glycine max*). Fourteen independent transgenic events introducing the *Pdex:AtDAD1-Myc* construct were identified, showing both basta resistance and PCR-amplification of the transgene (Figure S8a). In the absence of exogenous dex treatment, the oil extracts from leaves of all fourteen lines contained low background level of TAG similar to that in WT (Figure S8b upper panel);

however, upon dex treatment (12 h), several lines exhibited notable TAG accumulation (Figure S8b lower panel). Among the eight lines (indicated by arrows in the figure) that displayed visibly increased TAG compared to WT controls, one line (L7) was selected for quantitative analyses in the subsequent T2 generation. RT-qPCR showed 30-fold increase of *AtDAD1-Myc* transcript in dex-induced 3-wk-old L7 leaves compared to WT (Figure 7a). Increases in the transcript expression correlated with increased leaf TAG (~500 μg/gFW, equivalent to ~4 mg/g dry weight or 0.4% of leaf dry weight) (Figure 7b). The induction of TAG was also observable in older plants (6-wk-old) (Figure S9a) at the expense of galactolipids (Figure S9b), similar to Arabidopsis (Figures 3 and 4). Collectively, these results provide proof-of-concept for the biotechnological potential of using *DAD1* and *DAD1-like* genes to engineer crops to produce oil in leaves.

DISCUSSION

Conditional activation of plastid-localized DAD1 resulted in accumulation of TAG in leaves. Unlike seed TAG, the leaf TAG in the dex-induced *Pdex:AtDAD1-Myc* leaves featured a FA profile resembling that of leaf membrane lipids (Figure 5). A pathway model was drawn based on our observations and currently known metabolic pathways for TAG biosynthesis (Figure 1a). Galactolipids, especially the MGDG, are expected to be the major source providing FA building blocks for TAG assembly in the *Pdex:AtDAD1-Myc* leaves. This is because although AtDAD1 is classified as a phospholipase, galactolipids such as MGDG are likely its main substrate. This is conclusion is based on the predominance of galactolipids in the chloroplasts where AtDAD1 is localized, as well as reported substrate preferences for AtDAD1 (Ishiguro et al., 2001) and other plastidial PLA1s (Hyun et al., 2008; Kallenbach et al., 2010). Supporting this prediction, the increase in TAG was accompanied by concomitant decreases in MGDG and DGDG (Figure 4) while displaying no clear changes to most of phospholipid levels (Figure S2). A similar preference for MGDG was reported for an ABA-responsive plastidial lipase PLIP2 and a heat-inducible lipase (Higashi et al., 2018; Wang et al., 2018). In addition, MGDG is also the most abundant reservoir for 18:3 FAs which were found to be the most abundant FA component comprising the TAGs in the *Pdex:AtDAD1-Myc* leaves (Figure 5). However, with reference to the substrate preferences among different saturation levels of 18-carbon FAs, the results from

401 *Pdex:AtDAD1-Myc* / *fad3fad7fad8* plants (Figure S5b) (McConn and Browse, 1996; Miquel and
402 Browse, 1992) suggested that AtDAD1 can accept 18-carbon FA in the *sn1* position other than
403 18:3. Lack of 18:3 in *Pdex:AtDAD1-Myc* / *fad3fad7fad8* did not reduce overall TAG levels but
404 instead caused compensatory increases of TAG species with 18:2, 18:0 and 18:1 (Figure S5),
405 indicating that changes in saturation levels did not decrease AtDAD1 activity for lipid substrates
406 containing these FAs. AtDAD1's broad substrate specificity for lipid substrates with varying
407 saturation levels could be beneficial in engineering efforts aimed at tailoring FA composition for
408 different functionalities.
409
410 Upon hydrolysis from MGDG, 18:3 is expected to be exported from the chloroplast likely
411 through a similar mechanism as the FAs synthesized *de novo* involving FATTY ACID EXPORT
412 (FAX) proteins (Figure 1a) (Koo et al., 2004; Li et al., 2015). Subsequently, upon activation by
413 acyl activation enzymes (Koo et al., 2005; Zhao et al., 2019), the 18:3 will be added to the acyl-
414 CoA pool. These acyl-CoAs can then enter the TAG assembly pipeline either via the Kennedy
415 Pathway, where they sequentially combine with glycerol-3-phosphate molecules, or through the
416 Lands Cycle, where acyl-CoAs are joined to PC before incorporation into TAG (Bates et al.,
417 2007; Haslam et al., 2016). Nascent 18:1 leaving the plastid is first channeled into PC by the
418 lysophosphatidylcholine (LPC) acyltransferase (LPCAT) enzymes for acyl editing (Bates et al.,
419 2012; Karki et al., 2019). The 18:2 and 18 :3-CoA produced through acyl editing are then
420 utilized for glycerolipid assembly including PC which ultimately provides the DAG backbone
421 for TAG. Although there were no net changes in the overall PC levels (Figure S2), fluctuations
422 in several PC species with different acyl compositions have been observed in *Pdex:AtDAD1-*
423 *Myc*, indicative of an active acyl editing through PC (Figure S3). Next, two key enzymes,
424 DIACYLGLYCEROL ACYLTRANSFERASE1 (DGAT1) and
425 PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1 (PDAT1), were shown to
426 play major roles in the final steps of TAG assembly in leaves (Fan et al., 2013a; Tjellstrom et al.,
427 2015; Yurchenko et al., 2017). The relative contribution of the two pathways in incorporating the
428 AtDAD1-derived 18:3-CoA from plastid for TAG synthesis in the leaves of *Pdex:AtDAD1-Myc*
429 is unknown; however, there is some evidence supporting concerted action of another plastidial
430 lipase, PLIP1 and PDAT1 in Arabidopsis seeds (Aulakh and Durrett, 2019; Wang et al., 2017).
431 Additionally, the FA profile of leaf TAGs in PDAT1-overexpressing plants exhibited some

resemblance to that of *Pdex:AtDAD1-Myc*, with 18:3, 18:2, and 16:0 being the three dominant FAs, supportive of the contribution through PDAT1 (Fan et al., 2013a). In contrast, DGAT1-overexpression resulted in an increase in 18:1 and a reduction in 18:3 in TAG (Andrianov et al., 2010). Interestingly, transcripts of both *DGAT1* and *PDAT1* increased in *Pdex:AtDAD1-Myc* leaves 12 h after dex treatment (Figure S10). This upregulation may reflect positive feedback regulation of these genes at the transcript level rather than serving as a prerequisite for TAG synthesis, as TAG accumulation begins earlier at 4 h and 8 h post dex treatment (Figure 1b, Figure S1c). Meanwhile, this may suggest that the DGAT1 and/or PDAT1 activity might be limiting and that their co-expression with AtDAD1 in *Pdex:AtDAD1-Myc* could further enhance TAG accumulation. Ultimately, radiolabel pulse-chasing experiments (Johnson et al., 2024; Parchuri et al., 2024; Zhou et al., 2020) could provide detailed insights into the metabolic route of fatty acids from the chloroplast membrane to TAG in the cytosol.

A second metabolic fate of the 18:3 released by AtDAD1 in the plastid is octadecanoid pathway to synthesize oxylipins (Figure 1a) (Farmer and Ryan, 1992; Koo, 2018; Wasternack and Hause, 2013). This pathway has been shown to be primarily regulated at the level of substrate availability, namely by the provision of 18:3 (Kimberlin et al., 2022; Koo and Howe, 2009; Miersch and Wasternack, 2000; Scholz et al., 2015). Consistently, the induction of AtDAD1 in *Pdex:AtDAD1-Myc* by dex treatment led to accumulation of JA, its intermediary precursor 12-oxophytodienoic acid, and its downstream metabolites (Holtsclaw et al., 2024; Kimberlin et al., 2022). JA derivatives, particularly, its amino acid conjugate, jasmonoyl-isoleucine, serves as a hormonal signal for many stress responses and normal plant developmental processes such as fertility, making its synthesis essential for plant survival (Howe et al., 2018). However, the production of JA can also lead to plant growth inhibition (Poudel et al., 2016; Staswick et al., 1992; Zhang and Turner, 2008). AtDAD1 expressed under a JA-responsive promoter resulted in severe growth retardation (Kimberlin et al., 2022). Similar growth inhibitory effects were observed by the constitutive expression of AtDAD1 or PLIP2, another plastid-localized lipase involved in abscisic acid-induced JA biosynthesis (Ishiguro et al., 2001; Wang et al., 2018). Apart from the growth retardation through JA signaling, constitutive accumulation of leaf TAG could itself cause negative effects on growth (Fan et al., 2014; Vanhercke et al., 2019). In addition, plants with higher leaf TAG content were found to support greater insect growth

(Sanjaya et al., 2013; Yurchenko et al., 2018), potentially posing problems for the mass cultivation of high biomass oil lines in the field. However, these problems could possibly be mitigated by the inducible expression of AtDAD1, as *Pdex:AtDAD1-Myc* plants can be grown normally (Figure 3) until a desirable age when TAG accumulation can be induced. The production of lipid-derived defense signals such as JA by DAD1 may also enhance plant resistance against insects (Howe et al., 2018; Koo, 2018; Koo and Howe, 2009), although this remains to be tested. Induction of AtDAD1 led to largely equivalent TAG accumulation at various developmental stages except for the very young seedling (< 10-d-old) stage (Figure 3e). The time of AtDAD1 induction can be determined based on various considerations, including desirable biomass and flowering time. Harvesting the AtDAD1-induced leaves at the height of TAG accumulation can further reduce TAG loss through the turnover pathway involving TAG lipase and peroxisomal β-oxidation (Eastmond, 2006; Fan et al., 2014; Vanhercke et al., 2017; Yurchenko et al., 2017; Zolman et al., 2001). In addition, inducing DAD1 in senescent leaves (Kaup et al., 2002; Tjellstrom et al., 2015) or at specific time of the day (Gidda et al., 2016) may further promote TAG accumulation.

Oil accumulation was enhanced by mechanically wounding the *Pdex:AtDAD1-Myc* tissues that had been pretreated with dex (Figure S6). Wounding has been reported to induce TAG accumulation in leaves (Lewandowska et al., 2023; Vu et al., 2015; Vu et al., 2014), although this was not clearly observable under our current experimental conditions. However, co-treatment with dex and wounding in *Pdex:AtDAD1-Myc* leaves resulted in a greater increase in TAG levels than either treatment alone. A similar synergistic increase was observed for an 18:3-derived hormone, JA, following the same treatments (i.e., dex+wounding) (Kimberlin et al., 2022). However, JA increase was substantially more dramatic than TAG, with JA-levels increasing by 6-fold compared to either treatment alone. A post-transcriptional mode of regulations for the lipases (i.e, AtDAD1 or NbGLA1) by wounding has been postulated (Holtsclaw et al., 2024; Kimberlin et al., 2022). Why the same treatment does not result in as high TAG accumulation as JA is unclear, but the disparity might be, in part, due to the differences in their relative abundances- JA content in leaves is several orders of magnitude lower than that of lipids. It could also reflect a more streamlined conversion of 18:3 to JA by JA-metabolic enzymes than their assembly into TAGs, where there may be greater competition for

18:3 substrates by multiple enzymes, including those that incorporate FAs into membrane lipids. Regardless, from a biotechnological point of view, tissue damage that inevitably happens during crop harvest is expected to contribute positively to increasing leaf TAG yield.

Heterologous expression of AtDAD1 in *N. benthamiana* and soybean both resulted in similar increases in leaf TAGs, roughly estimated to be about 0.4% of dry leaf mass. The findings demonstrate the translational potential of this technology to crop plants. While 0.4% is modest compared to some of the highest achieving lines, such as transgenic *Nicotiana tabacum* lines that accumulated TAG up to 30% of dried leaf weight (Vanhercke et al., 2017), similar modest increases have been reported in several engineering efforts (Cai et al., 2017; Sanjaya et al., 2013; Yang et al., 2015; Yurchenko et al., 2017). ThePLA1-based approach presented is likely compatible with and expected to become more effective when combined with existing lines developed to enhance vegetative oil content. Although PLA genes have diverged into a large number in higher plants (Ali et al., 2022; Kelly and Feussner, 2016; Ryu, 2004; Wang, 2001), the well-conserved nature of lipase domains and the broad substrate specificities make it likely that the ectopic expression of homologous genes will result in similar effects as AtDAD1. Supporting this concept, transient expression of *NbDAD1* in *N. benthamiana* leaves caused largely equivalent increases of leaf TAG as *AtDAD1* (Figures 6 and S7); however, characterization is needed to determine whether this process follows similar metabolic pathways of converting FAs originated from the plastidial galactolipids to the TAGs in the cytosol as with AtDAD1 in Arabidopsis. In addition, further investigation is required to determine whether the presented increase in leaf TAG by PLAs is limited to PLAs localized in plastids or can be extended to extra-plastidial PLAs. Given the general observation of TAG accumulation in leaves by diverse stress conditions that are likely to be attributed to induction of diverse PLAs suggests the latter possibility, i.e., contribution by extra-plastidial PLAs to stress-induced TAG accumulation (Higashi et al., 2018; Kelly and Feussner, 2016; Lewandowska et al., 2023; Rajashekar et al., 2006; Tan et al., 2018; Vanhercke et al., 2019; Welti et al., 2002; Yang et al., 2011).

MATERIALS AND METHODS

Plant materials and chemicals

Arabidopsis (*Arabidopsis thaliana*) was cultivated under long-day conditions (16 h light) with a light intensity of 100-120 μE m^{-2} s^{-1} in growth chambers maintained at 22 °C. Columbia-0 (Col-0) was used as the wild-type (WT). Arabidopsis seeds were either directly sown in soil or initially grown on solid Linsmaier and Skoog (LS) media (Caisson Laboratories, UT, USA) (0.7% w/v Phytoblend agar, 0.7% w/v sucrose) with or without antibiotics before being transferred to soil. *Nicotiana benthamiana* was grown in a chamber kept at 22°C with a 16-h-light photoperiod with a light intensity of 130-150 μE m^{-2} s^{-1}. Soybean (*Glycine max*) cultivar 'Maverick' was grown in an environmental chamber set at day/night cycle of 25 °C/22 °C with a 16 h-light photoperiod with 120-150 μE m^{-2} s^{-1} intensity light.

Mechanical wounding of Arabidopsis leaves was performed on 3-week-old plants as previously described (Herde et al., 2013). All tissue samples were flash frozen in liquid nitrogen upon harvest and stored at -80 °C until use. For oil, protein, and nucleic acid analyses, the frozen tissues were pulverized to a fine powder in 2 mL screw-capped microcentrifuge tubes containing metal beads using a tissue homogenizer (TissueLyser II, Qiagen, Hilden, Germany) immediately before extractions.

Dexamethasone (dex), α-linolenic acid ((9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid) (α-LA), pentadecanoic acid, heptadecanoic acid, tripentadecanoin, triheptadecanoin, ammonium glufosinate, Nile Red, and primulin were purchased from MilliporeSigma (Burlington, MA, USA). BODIPY (493/503) was from Cayman Chemical (Ann Arbor, MI, USA).

Transgenic lines and transient expression

The cloning of dex-inducible vector constructs *Pdex:AtDAD1-Myc* and its active site-mutated variant *Pdex:AtDAD1^{mut}-Myc*, along with the generation of their respective Arabidopsis transgenic lines, has been described previously (Kimberlin et al., 2022). The *Pdex:AtDAD1-Myc* gene construct was also introduced into the *fad3fad7fad8* triple mutant background (McConn and Browse, 1996) using the Agrobacterium-mediated floral dip method (Clough and Bent, 1998). Sterile flowers of JA-deficient *fad3fad7fad8* plants were thoroughly sprayed with a solution containing 100 μM methyl-JA (MilliporeSigma) to induce fertility once every day from 3 d before floral dipping, continuing for another 5 d.

The *Pdex:NbDAD1* construct was generated by PCR-amplifying *NbDAD1* (Niben101Scf02386g01005.1) from cDNA prepared from *N. benthanmiana* leaf tissues using

Phusion High-Fidelity Polymerase (New England Biolabs, Ipswich, MA) and primers described in Table S2. The resulting PCR fragment was initially cloned into the pGEM-T Easy vector system (Promega, Madison, WI). Upon sequence verification, the *NbDAD1* insert was subcloned into the glucocorticoid-inducible vector system (*Pdex*) (Aoyama and Chua, 1997; Koo et al., 2009), utilizing *XhoI* as the restriction enzyme site. Transient expression in *N. benthamiana* leaves was conducted using C58C1 strain of *Agrobacterium tumefaciens* carrying *Pdex:AtDAD1-Myc, Pdex:NbDAD1*, or empty vector constructs according to previous described protocol (Holtsclaw et al., 2024). To induce gene expression, an induction solution containing dex (30 μM in 0.01% Triton X-100 in water) was sprayed to saturation on the adaxial side of the leaf. For *N. benthamiana*, 11-mm diameter leaf discs were punched from the surrounding regions of the syringe-infiltration site after the dex spraying.

Transgenic soybean lines expressing *Pdex:AtDAD1-Myc* were generated at the Transformation Core Facility, University of Missouri. Fourteen transgenic events (designated as L1-L14) were obtained as described in the Results (Figure S8).

RNA analysis

Total RNA was extracted from samples containing 50-100 mg of pulverized frozen tissue powders using TRIzol reagent (Thermo Fisher, MA, USA) and the Direct-zol RNA MiniPrep Plus Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. cDNA was reverse transcribed from 1 μg of total RNA using the iScript Reverse Transcription Supermix (BioRad, Hercules, CA, USA) and oligo $(dT)_{20}$ primers. The resulting cDNA served as a template for either semi-quantitative reverse transcriptase PCR (RT-qPCR) with the iTaq SYBR Green Supermix (BioRad) in a CFX96 Touch real-time PCR detection system (BioRad), or regular reverse transcription PCR (RT-PCR) using Bioline BioMix Red (Meridian Bioscience, London, UK). *ACT8* (At1g49240), *NbEF1α* (Niben101Scf08653g00001.1) (Kallenbach et al., 2010), and *GmACT2* (GenBank: AW350943) (M. Libault, 2008) were used as the internal reference genes for Arabidopsis, *N. benthamiana*, and *G. max*, respectively, using primers listed in Table S2.

Protein extraction and Western blot analysis

Proteins were extracted from around 100 mg of ground frozen tissues according to a previously described procedure (Kimberlin et al., 2022). An aliquot of the total protein extract was used to determine protein concentration using the Bradford Assay (BioRad, Hercules, CA, USA). Twenty μg of total protein was separated on a 10% SDS-PAGE gel. Before loading, samples were mixed with sample buffer (6 M urea in 2×Laemmli buffer) and incubated at 37 °C for 30 min. For Western blot analysis, proteins in SDS-PAGE gels were transferred to polyvinylidene difluoride membranes and probed with polyclonal antibodies against *Myc* (Abcam, Cambridge, UK) at a 1:3000 dilution, followed by incubation with a secondary antibody (anti-rabbit horseradish peroxidase (HRP), MilliporeSigma) at a 1:15,000 dilution. The HRP signal was detected by X-ray film exposure in the presence of a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent, Thermo Fisher Scientific, MA, USA).

Lipid extractions, thin layer chromatography, and derivatization

Lipid extraction for thin-layer and gas chromatographic analysis followed a previously described method (Hara and Radin, 1978) with minor modifications (Koo et al., 2005; Koo et al., 2004). Tissue samples for oil analyses were taken from leaves of 24-d-old Arabidopsis, 3- or 6-wk-old soybean, and 4-wk-old *N. benthamiana* plants, as described in each figure legends. Approximately 200 mg of frozen tissue, ground to a fine power, was immediately placed in 4 mL of pre-warmed (90 °C) isopropanol containing internal standards (15:0 FA, 17:0 FA, tripentadecanoin or triheptadecanoin) with 100 μg of butylated hydroxytoluene (BHT) and incubated for 15 min at 90 °C. The cooled sample was mixed with hexane (6 mL) and 15% aqueous sodium sulfate (5 mL). The upper phase was collected and combined with subsequent extractions using 1.5 mL of hexane followed by another 4 mL of isopropanol/hexane (v/v = 2/7). The pooled extracts were dried down under streams of nitrogen gas and resuspended in 0.2-1 mL of hexane (for neutral lipid analysis) or acetone (for total lipid analysis).

For neutral lipid separations, lipid extracts were loaded onto thin layer chromatography (TLC) Silica Gel 60 plates (MilliporeSigma, Burlington, MA, USA) and developed using hexane/diethyl ether/acetic acid (v/v/v = 80/20/1) as the mobile phase. For polar lipid separation, TLC Silica Gel 60 plates were submerged in 0.15 M ammonium sulfate solution and allowed to dry completely. The dried plates were activated by baking in 120 °C oven for 2 h immediately prior to use. A solvent mixture consisting of acetone/toluene/water (v/v/v = 91/30/7.5) was used

as the mobile phase for polar lipid separation. Lipids on TLC plates were visualized by either briefly placing in a sealed tank containing iodine crystals or spraying with 50% sulfuric acid followed by charring at 120 °C for 15 min. The TLC plates with lipids for subsequent recovery and GC analysis was stained with primulin (0.05% in acetone/water (v/v = 8/2)) (White et al., 1998).

For GC analysis, TLC bands containing lipids were scraped from the plate and extracted by multiple iterations of sonication (15 min) with hexane and chloroform for TAG and a mixture of chloroform/methanol/water (v/v/v = 5/5/1) for galactolipids and phospholipids. Resulting lipid extracts were dried down under stream of nitrogen gas and resuspended in 100 µL toluene. Derivatization to FAMEs was carried out by incubating at 90 °C for 45 min in 1 mL of methanolic boron trifluoride solution (MilliporeSigma, Burlington, MA, USA). After samples were cooled, 1 mL of water was added, and FAMEs were extracted three times with 3 mL hexane and concentrated.

For lipidomics analysis, lipids were extracted by placing whole leaf tissues (200-300 mg) into 4 mL of hot isopropanol (75 °C) containing 0.01% BHT and incubating for 15 min. To cooled samples, 12 mL of chloroform/methanol/water (v/v/v = 30/41.5/3.5) was added and shaken at 100 rpm for 24 h. Lipid extracts were dried down under nitrogen gas and resuspended in 1 mL chloroform. Tissue materials were recovered, dried, and weighed.

Lipid analysis by GC-FID and ESI-LC-MS/MS

Transmethylated lipids resulting in FAMEs were analyzed using a Focus GC gas chromatograph flame ionization detector (GC-FID, Thermo Scientific, MA, USA) with an installed DB-23 capillary column (Agilent Technologies, CA, USA) and XCalibur control software as previously described (Morley et al., 2023; Koley et al., 2022). Briefly, conditions for the GC-FID were as follows; sampling volume (µL): 2, split mode flow (mL/min): 20, inlet temperature: 250 °C, FID temperature: 250 °C, carrier gas constant flow (mL/min): 2. Gradient conditions during a sample injection included an initial temperature: 170 °C held for 1 min, temperature gradient (°C/min): 10, final temperature: 250 °C held for 2 min. Integration of GC-FID peaks was performed using XCalibur software. FAMEs with chain lengths of 15:0, 16:0, 17:0, 18:0, 18:1, 18:2, 18:3, 20:0, 20:1, 20:2, and 22:1, were detected with 15:0 or 17:0 serving as an internal standard for quantitation. Peak detection was performed using the Genesis algorithm, using the nearest

assigned retention time for each FAME and a signal/noise ratio greater than 3. Retention times were previously established through analysis of standards. Integration of detected peaks used 1 smoothing point and a signal/noise threshold of 0.5. The integration of all peaks was inspected and peaks that were small or with poor quality peak shape were manually integrated as necessary.

Electrospray tandem triple quadrupole mass spectrometry (ESI-MS/MS) analyses were performed by direct infusion into an Applied Biosystems 4000 QTrap (Sciex, Framingham, MA, USA) with an electrospray ionization source, and lipid mass spectral parameters are indicated in Table S3. Polar lipid analysis was similar to that described (Xiao et al., 2010) in their supplemental data. After calculation of polar lipid values in relation to the internal standards, phospholipid values were corrected using response factors calculated for the employed internal standards vs the SPLASH Lipidomix (product 330707, Avanti Polar Lipids, Alabaster, AL, USA), and galactolipids were corrected using response factors determined by comparing data from gas chromatographic analysis of FAMEs from MGDG and DGDG with data on intact lipids analyzed by direct infusion lipidomics as done in this work. TAG analysis was performed as previously described (Li et al., 2014). However, no response factors were employed, and data are presented in signal units, in which a unit of 1 is equal to the signal of 1 nmol of internal standard.

Histochemical staining of lipid droplets and confocal microscopy

Lipid droplets (LDs) were stained with BODIPY493/503 (Cayman Chemical, Ann Arbor, MI) or Nile Red (MilliporeSigma, Burlington, MA, USA). Tissues samples of 16-d-old leaves and roots or 6-wk-old plants were incubated in 50 mM PIPES (pH 7.0) containing 0.004 mg/mL BODIPY or 6.5 mg/mL Nile Red for 5-30 min before washing with 50 mM PIPES (pH 7.0). As a control, tissues samples were pre-incubated in a 100 μM α-LA solution for 1 h prior to lipid staining. Images were acquired using a Leica TCP SP8 STED confocal microscope featuring a Leica 633 Plan Apochromat oil-immersion objective (40×) or a dry objective (10×), and the Leica Application Suite X (LAS X) package. BODIPY, Nile Red and chlorophyll autofluorescence were activated using a 488-nm laser with an adjusted pinhole set to 3. Emission fluorescence signals were gathered within the ranges of 501-506 nm for BODIPY, 528-650 nm for Nile Red, and 680-750 nm for chlorophyll.

ACCESSION NUMBERS

Accession numbers

Accession numbers for genes appearing in this paper are as follows from Arabidopsis.org, National Center for Biotechnology Information library, and Sol Genomics Network (genome release, v1.0.1). *DAD1* (At2g44810, AEC10469.1); *DGAT1* (At2g19450); *PDAT1* (At5g13640), *NbDAD1* (Niben101Scf02386g01005.1); *ACT8* (At1g49240); *NbEF1α* (Niben101Scf08653g00001.1); *GmACT2* (GenBank: AW350943).

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data discussed in this study can be found in the article and in the Supplementary Materials.

SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article:

Table S1. Lipidomics analysis data for leaf polar lipids of WT, *Pdex:AtDAD1-Myc*, and *Pdex:AtDAD1-Myc* / *fad3fad7fad8* plants.

Table S2. Lipidomics analysis data for leaf TAGs of WT, *Pdex:AtDAD1-Myc*, and *Pdex:AtDAD1-Myc* / *fad3fad7fad8* plants.

Table S3. DNA primers used in this study.

Table S4. Mass spectrometry parameters for lipidomics. Lipidomics analysis was performed as described in the Methods.

Figure S1. Induction of AtDAD1 expression leads to oil accumulation in Arabidopsis leaves.

Figure S2. Phospholipids (PLs) in the leaves of dex-induced *Pdex:AtDAD1-Myc* plants.

Figure S3. Lipidomics analysis of individual PL species in the leaves of dex-induced *Pdex:AtDAD1-Myc* plants.

Figure S4. Effects of *AtDAD1-Myc* expression on unsaturation index of various lipids classes of WT and *Pdex:AtDAD1-Myc*.

Figure S5. Ectopic expression of *AtDAD1-Myc* in *fad3fad7fad8* leaves leads to increase of TAGs.

Figure S6. Effect of mechanical wounding on leaf TAG accumulation.

Figure S7. Leaf TAG increases at the expense of galactolipids in *N. benthamiana* leaves expressing *AtDAD1-Myc*.

Figure S8. Development of transgenic soybean (*Glycine max*) lines with inducible leaf oil.

Figure S9. TAG increases in 6-wk-old transgenic soybean (*Glycine max*) leaves.

Figure S10. Expression of *DGAT1* and *PDAT1* in Arabidopsis *Pdex:AtDAD1-Myc* leaves.

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FIGURE LEGENDS

Figure 1. AtDAD1-dependent TAG accumulation in Arabidopsis leaves. (a) A model of plastidial PLA1-induced TAG synthesis in leaves. Induction of PLA1 expression by either stressors or an inducible-vector system leads to the hydrolysis of membrane lipids to generate free FAs, primarily 18:3. The free FAs are integrated into the acyl-CoA pool and subsequently

incorporated into TAG via either the Kennedy Pathway or the Lands Cycle. A portion of the 18:3 is converted to oxylipins. (b-c) Quantification of TAG (b) and total FAME (c) in fully expanded rosette leaves of 24-d-old WT and *Pdex:AtDAD1-Myc* treated with dex for the shown durations. (d-e) TLC image (d) and quantification (e) of TAGs in leaves of 24-d-old WT or transgenic lines expressing either intact AtDAD1-*Myc* or its mutant variant with the catalytic site substituted with Ala (*AtDAD1*^{mut}). Polar lipids at the origin display equal loading (d). Bar graphs represent means ± SD of three biological replicates. Asterisks indicate statistical significance (Students' *t* test, *P<0.05, **P<0.01) compared to WT.

Figure 2. Lipid droplets (LDs) accumulate in vegetative tissues of Arabidopsis *Pdex:AtDAD1-Myc*. (a) Confocal fluorescence micrographs of leaf mesophyll cells in 16-d-old WT or *Pdex:AtDAD1-Myc* treated with mock (0.01% Triton X-100 in water) or dex (30 μM in 0.01% Triton X-100) for 8 or 12 h. Red shows chlorophyll autofluorescence (650-750 nm) whereas LDs stained with BODIPY (503 nm) or Nile Red (495-550 nm) are shown in green. Scale bars are 20 μm. (b) BODIPY staining of lipid droplets in 6-wk-old (petiole and stem) or 16-d-old (root) WT or *Pdex:AtDAD1-Myc* treated with dex (30 μM, 12 h). Cross-sectional view of stem is shown. Petioles and roots were not sectioned. As a control, WT tissues were incubated with α-LA (100 μM) for 1 h. Fluorescent images were overlayed to transmitted light image for stem and root. Scale bars are 100 μm (petiole and stem) or 20 μm (root).

Figure 3. TAG-inducibility in the Arabidopsis leaves at multiple developmental stages. (a-b) Photo (a) and fresh mass (b) gain of the arial part of soil-grown WT and *Pdex:AtDAD1-Myc* plants over days (8-35 d) without dex, exhibiting no statistical differences. (c-d) RT-qPCR (c) and immunoblot (d) detection of *AtDAD1-Myc* transcripts and proteins at various plant ages after 8 h treatment with either mock (0.01% Triton X-100 in water) or dex (30 μM in 0.01% Triton X-100). Graphs represent mean ± SD of five (b) or three (c) biological replicates. Letters above bars denote statistical significance (pairwise *t*-tests, $P < 0.05$). *ACT8* was used as a reference and the relative transcript level values are based on comparisons to the mock (no dex). Two specific bands detected on the immunoblot using anti-*Myc* antibody are likely the precursor (P) and the mature (M) forms of AtDAD1-*Myc* before and after cleavage of the chloroplast transit peptide (d) as reported previously (Kimberlin et al., 2022). Coomassie stain of rubisco band shows the

protein loading. (e) Sulfuric acid charred TLC plate showing relative TAG production throughout the indicated development time course.

Figure 4. Leaf TAG increases at the expense of galactolipids in Arabidopsis *Pdex:AtDAD1-Myc* plants. (a) TLC plate showing decrease of MGDG and DGDG over time with induction of *AtDAD1-Myc*. Fully expanded rosette leaves of 24-d-old *Pdex:AtDAD1-Myc* plants were sprayed with 30 μM dex for indicated times. WT leaves were equally treated and incubated for 12 h. Total lipids were separated on TLC impregnated with ammonium sulfate using acetone/toluene/water (91/30/7.5) as the mobile phase. Lipids were visualized by spraying with a sulfuric acid solution followed by charring the plates at 120 °C. (b-c) Quantification of MGDG (b) and DGDG (c) using GC-FID. (d-e) Lipidomics analysis of MGDG (d) and DGDG (e). Lipidomics analysis was carried out as described in the Methods. Graphs are mean ± SD of three biological replicates. Asterisks denote statistical significance compared to WT control (Student *t*-tests, *$P < 0.05$).

Figure 5. Fatty acid composition of TAG in Arabidopsis *Pdex:AtDAD1-Myc* leaves. (a) FAME analysis of TAG increases over time in 24-d-old *Pdex:AtDAD1-Myc* leaves upon induction by dex. (b) Comparison of FAME compositions in total lipids, MGDG, DGDG and TAG of WT and *Pdex:AtDAD1-Myc* after 12 h of dex treatment. (c) Lipidomics analysis of TAG species. Total lipid extracts were subjected to LC-MS analysis as described in the Methods. Data are mean (b) or mean ± SD (a and c) of three biological replicates.

Figure 6. Production of TAG in *N. benthamiana* leaves by ectopic expression of *AtDAD1-Myc* and *NbDAD1*. (a) RT-PCR detection of *AtDAD1* and *NbDAD1* transcripts. *NbEF1α* was used as a reference. (b-c) TLC separation of neutral lipids (b) and quantification of leaf TAG by GC-FID (c). Multiple lanes per gene construct indicate biological replicates (a and b). Four-week-old *N. benthamiana* leaves were syringe-infiltrated with Agrobacteria strains carrying empty vector (*EV*), *Pdex:AtDAD1-Myc,* or *Pdex:NbDAD1* plasmid constructs for 2 days. Dex (30 μM) solution was sprayed and tissues were collected after 6 h. Total lipids were separated using a hexane/diethylether/acetic acid (80/20/1) and subsequently charred after spraying with a sulfuric acid solution. Graph represents median value with maximum and minimum data values of three biological replicates, with letters above the bars denoting statistical significance (pairwise *t*-tests, $P < 0.05$).

Figure 7. Development of transgenic soybean (*Glycine max*) lines with increased leaf TAG contents. (a) RT-qPCR analysis of transgene (*AtDAD1*) transcripts in soybean line 7 (L7) transformed with *Pdex:AtDAD1-Myc*. (b) Quantification of TAG in *G. max* WT and L7 leaves. Three-week-old leaves were sprayed with mock (0.01% Triton X-100 in water) or dex (30 μM, 0.01% Triton X-100) for 12 h. *GmACT2* was used as a reference (a) and TAG quantification was by GC-FID (b). Bar graphs represent mean ± SD of three biological replicates, with letters above the bars denoting statistical significance (pairwise *t*-tests, $P < 0.05$)