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ORIGINAL ARTICLE

The effect of *AINTEGUMENTA-LIKE 7* over-expression on seed fatty acid biosynthesis, storage oil accumulation and the transcriptome in *Arabidopsis thaliana*

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Abstract

Key message ***AIL7*** **over-expression modulates fatty acid biosynthesis and triacylglycerol accumulation in** ***Arabidopsis*** **developing seeds through the transcriptional regulation of associated genes.**

Abstract Seed fatty acids (FAs) and triacylglycerol (TAG) contribute to many functions in plants, and seed lipids have broad food, feed and industrial applications. As a result, an enormous amount of attention has been dedicated towards uncovering the regulatory cascade responsible for the fine-tuning of the lipid biosynthetic pathway in seeds, which is regulated in part through the action of LEAFY COTYLEDON1, ABSCISSIC ACID INSENSITIVE 3, FUSCA3 and LEC2 (LAFL) transcription factors. Although AINTEGUMENTA-LIKE 7 (AIL7*)* is involved in meristematic function and shoot phyllotaxy, its effect in the context of lipid biosynthesis has yet to be assessed. Here, we generated *AIL7* seed-specific over-expression lines and found that they exhibited significant alterations in FA composition and decreased total lipid accumulation in seeds. Seeds and seedlings from transgenic lines also exhibited morphological deviations compared to wild type. Correspondingly, RNA-Seq analysis demonstrated that the expression of many genes related to FA biosynthesis and TAG breakdown were significantly altered in developing siliques from transgenic lines compared to wild-type plants. The seed-specific over-expression of *AIL7* also altered the expression profiles of many genes related to starch metabolism, photosynthesis and stress response, suggesting further roles for *AIL7* in plants. These findings not only advance our understanding of the lipid biosynthetic pathway in seeds, but also provide evidence for additional functions of *AIL7*, which could prove valuable in downstream breeding and/or metabolic engineering endeavors.

Keywords AINTEGUMENTA-LIKE 7 · Lipid · Fatty acid · Gene over-expression · Transcriptomics · Plant development

Introduction

Triacylglycerol (TAG), which constitutes three fatty acids (FAs) esterified to a glycerol backbone, is a major storage compound in eukaryotic cells. In plants, TAG is mainly stored in seeds and fruits, and is utilized for a variety of functions including seedling growth during germination, pollen development, and sexual reproduction. Plant-derived oils, and particularly seed oils, also have enormous economic importance due to their popularity for food, feed, and industrial applications, as well as growing interest in their use as a renewable feedstock for biodiesel and non-petroleum-based biomaterial production (Lu et al. 2011; Singer and Weselake 2018). While the majority of seed oils comprise predominantly palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1Δ^{9cis}; hereafter C18:1), linoleic acid (C18:2$\Delta^{9cis,12cis}$; hereafter C18:2), and α-linolenic acid (C18:3$\Delta^{9cis,12cis,15cis}$;

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hereafter C18:3), the precise proportions of these FAs can differ quite substantially depending on the species, providing each oil with unique functional properties that govern their potential for particular downstream applications (Jain 2020).

Due to an escalating demand for seed oils with various functionalities, a substantial amount of research has been directed towards improving seed lipid-related traits over the last few decades by manipulating crop genetics via breeding or biotechnology (e.g., Katche et al. 2019; Msanne et al. 2020; Subedi et al. 2020). To aid in this endeavor, considerable efforts have been undertaken to better understand the lipid biosynthetic pathway at the molecular level (e.g., Gao et al. 2021; Singer et al. 2016; Woodfield et al. 2019). However, due to the extremely multifaceted nature of lipid biosynthetic pathways in plants, and the correspondingly large number of genes involved, gaps remain in our understanding of this process.

During seed development, sucrose derived from photosynthesis serves as a carbon source for the synthesis of different storage compounds, including TAG. In the case of TAG biosynthesis, incoming sucrose is converted to glycolytic intermediates within the seed, where they are utilized for the synthesis of acetyl-coenzyme A (CoA), which serves as a precursor for the de novo biosynthesis of various FAs in plastids. The termination of de novo FA biosynthesis can be catalyzed by either plastidial acyl-ACP acyltransferases, which use acyl-ACP directly for the production of glycerolipids in the plastid, or acyl-ACP thioesterases, which catalyze the hydrolysis of acyl-ACP and result in the release of free FAs (typically C16:0, C18:0 and C18:1) and ACP. These free FAs are then transferred to the outer plastid envelope and are re-esterified to CoA, after which time they enter the cytosolic pool of acyl-CoA, are transported to the endoplasmic reticulum (ER), and can undergo further modifications (desaturation, elongation). In higher plants, C18:1 can be further desaturated to C18:2 and C18:3 through the catalytic action of the ER-bound fatty acid desaturase 2 (FAD2) and FAD3, respectively (Subedi et al. 2020). Elongation of C16 and C18 FAs to chain lengths of 20 carbons or greater, on the other hand, is catalyzed by the ER-bound fatty acid elongase (FAE) complex, consisting of four enzymatic reactions including 3-ketoacyl-CoA-synthetase [FAE1; (Huai et al. 2015)]. In plants, TAG can then be synthesized de novo through the sequential acyl-CoA-dependent acylation of a glycerol backbone on the ER [Kennedy pathway; (Weiss et al. 1960)], or through an acyl-CoA-independent pathway that uses phosphatidylcholine as the acyl donor and *sn*-1, 2-diacylglycerol as the acceptor (Dahlqvist et al. 2000).

Several transcription factors (TFs) have been shown to play important roles in the regulation of FA biosynthesis/modification and seed lipid accumulation. For example, LEAFY COTYLEDON1 (LEC1), LEC2, ABSCISIC ACID INSENSITIVE3 (ABI3) and FUSCA3 (FUS3) are known to act as master regulators of lipid biosynthesis during embryogenesis and seed maturation, and various other TFs, such as WRINKLED1 (WRI1), MYB96 and bZIP67, have also been found to play a role (Kong et al. 2019, 2020a, b; Kumar et al. 2020). However, the functions of many TF-encoding genes that are expressed during the maturation phase of seed development when TAG is known to accumulate have yet to be elucidated in the context of lipid-related pathways during seed development/maturation (Baud et al. 2008; Weselake et al. 2009). One such gene is *AINTEGUMENTA-LIKE 7* (*AIL7*; also known as *PLETHORA7* [*PLT7*]; AT5G65510), which is a member of the AIL/PLT subfamily of the large APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain TF family (Krizek 2015). The AIL/PLT subfamily comprises 8 genes in *Arabidopsis*, including *AINTEGUMENTA* (*ANT*), *BABY BOOM* (*BBM*), *PLT1*, *PLT2*, *AIL6*/*PLT3*, *AIL5*/*PLT5, AIL7*/*PLT7* and *AIL1*, which encode proteins that share a relatively high level of amino acid identity within their AP2 domains, and in certain cases between their full-length protein sequences (e.g., 74% amino acid similarity between AIL6 and AIL7; Nole-Wilson et al. 2005). Genes within the AIL/PLT subfamily are expressed in dividing tissues where they have overlapping functions in various shoot, floral and root developmental processes (Horstman et al. 2017, 2014; Mudunkothge and Krizek 2012; Nole-Wilson et al. 2005). While single *ail7* Arabidopsis mutants have not been found to exhibit any obvious morphological or developmental defects due to functional redundancy with other member of the AIL/PLT subfamily (Mudunkothge and Krizek 2012; Prasad et al. 2011), triple *ant*/*ail6*/*ail7* mutants display severe aberrations in apical meristem activity (Mudunkothge and Krizek 2012), triple *ail5*/*ail6*/*ail7* mutants exhibit altered phyllotaxis (Prasad et al. 2011) and lateral root outgrowth (Du and Scheres 2017), and *ant*/*ail7* double mutants exhibit alterations in flower development (Krizek 2015).

Several other TFs belonging to the AP2/ERF family are known to modulate seed oil biosynthesis indirectly via a role in sugar metabolism. For example, *ap2* mutants have been found to produce large seeds with concomitant increases in seed oil content, which has been suggested to occur, at least in part, through the alteration of source-sink relations and soluble sucrose metabolism in developing seeds (Jofuku et al. 2005; Ohto et al. 2005). Direct roles in seed oil biosynthesis have also been observed for members of this gene family, including WRI1, which functions as a major transcriptional regulator of fatty acid biosynthesis in seeds (Baud et al. 2007; Kong et al. 2020a, b). While members of the *AIL* subfamily have not been implicated in FA/TAG biosynthesis or accumulation as of yet, several (including *AIL7*) are expressed during embryo development at a time consistent with TAG deposition (Le et al. 2010) and both BBM and PLT2 have been found to regulate the expression

of the LAFL network in Arabidopsis (Horstman et al. 2017). Furthermore, although *AIL7* has not been found to be required for the establishment of the shoot apical meristem during embryogenesis (Mudunkothge and Krizek 2012), like BBM and AIL5 its constitutive over-expression in *Arabidopsis* leads to the production of somatic embryos (Horstman et al. 2017), suggesting that it may play a role in embryonic development.

Given its putative and as of yet un-deciphered role in the embryo, we sought to determine the function of *AIL7* in the context of seed development and storage TAG biosynthesis/accumulation. To achieve this, we developed seed-specific *AIL7* over-expression Arabidopsis lines, which can circumvent issues related to possible functional redundancy with other AIL/PLT subfamily members, and carried out subsequent morphological characterization, as well as TAG content and composition analyses. We also compared the gene expression profiles of the over-expression and wild-type lines using RNA-Seq to provide further insight into the regulatory functions of *AIL7* during seed development. Our findings provide evidence for novel storage TAG-related functions for *AIL7*, including the regulation of acyl modification and seed TAG accumulation, and also hint at additional putative roles for this TF in processes, such as starch metabolism, stress response and photosynthesis.

Materials and methods

RNA extraction and first-strand cDNA synthesis

Siliques were harvested from *Arabidopsis* plants (Col-0 background) at various [14 days after flowering (DAF)], flash-frozen in liquid nitrogen, and stored at – 80 ºC until further use. Total RNA was extracted using the Sigma Spectrum Plant Total RNA kit (Sigma-Aldrich Canada Co., Oakville, ON) and any contaminating DNA was removed using the manufacturer's on-column DNase set (Sigma-Aldrich). First-strand cDNA synthesis was carried out using the Superscript III first-strand cDNA synthesis kit according to the manufacturer's instructions (Thermo Fisher Scientific, Inc., Waltham, MA) with 250 ng RNA as template along with an oligo(dT) primer.

Generation of the *AIL7* seed-specific over-expression construct

The *AIL7* seed-specific over-expression construct (AIL7-OE) was generated by first amplifying the full-length 1497-nt *AIL7* coding region (AT5G65510) from cDNA derived from Col-0 developing siliques (14 DAF) using Platinum High Fidelity polymerase (Thermo Fisher Scientific, Inc.) and primers AtAIL7F1AgeI (5′—CTA **CCG GT**A TGG CTC CTC CAA TGA CG—3′) and AtAIL7R1BamHI (5′—CCA **GGA TCC** TTA GTA AGA CTG GTT AGG—3′), which contained restriction sites near their 5′ ends to facilitate cloning (shown in bold). Thermal parameters for amplification were as follows: 94 ºC for 2 min, 32 cycles of 94 ºC for 15 s, 55 ºC for 30 s and 68 ºC for 2 min, followed by a final extension of 68 ºC for 7 min. The resulting fragment was inserted between the seed-specific *Brassica napus napin* promoter (Josefsson et al. 1987) and *Pisum sativum Ribulose-1,5-bisphosphate carboxylase* transcriptional terminator (*rbcS-t*) in a pGreen 0029 background [(Hellens et al. 2000); Fig. 1A]. Sequencing was carried out at every step of plasmid construction to ensure the correct identity of the resulting plasmid.

Arabidopsis transformation and plant growth conditions

The AIL7-OE vector was introduced into *Agrobacterium tumefaciens* strain GV3101 via electroporation along with the pSoup helper plasmid (Hellens et al. 2000). The resulting recombinant bacteria were utilized for the transformation of *Arabidopsis* Col-0 plants using the floral dip method (Clough and Bent 1998), and first-generation transformants were selected by plating surface-sterilized seeds

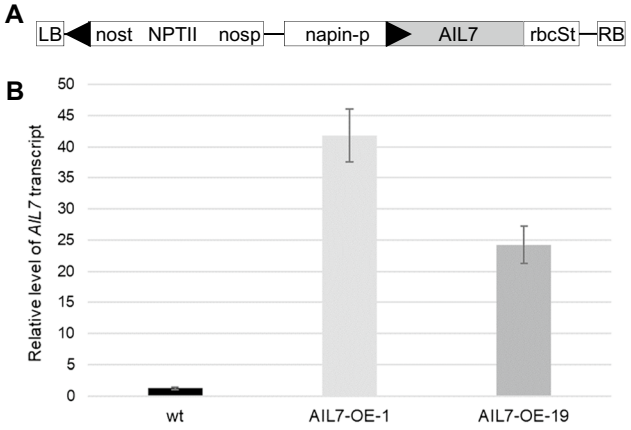

Fig. 1 Generation of seed-specific *AIL7* over-expression *Arabidopsis* lines. **A** Schematic representation (not to scale) of the AIL7-OE construct used in this study. Arrows indicate the direction of transcription. **B** Quantitative real-time RT-PCR analysis of *AIL7* expression in T_2 siliques (14 DAF) from AIL7-OE-1, AIL7-OE-19, and wild-type lines. Blocks denote mean *AIL7* transcript levels from three biological replicates relative to the internal control, *PP2AA3*. Three technical replicates were carried out for qRT-PCR assays and bars indicate standard errors. *AIL7 Arabidopsis AINTEGUMENTA 7* coding sequence, *LB* left T-DNA border, *napin-p Brassica napus napin* promoter, *nosp NOPALINE SYNTHASE* promoter, *nost NOPALINE SYNTHASE* transcriptional terminator, *NPTII*, *NEOMYCIN PHOSPHOTRANSFERASE II*, *RB* right T-DNA border, *rbcSt Pisum sativum RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE* transcriptional terminator, *wt* wild-type

on half-strength Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 1% sucrose, 0.8% agar, 100 mg/l timentin, and 50 mg/l kanamycin. Transgenic T_1 and T_2 seedlings were transferred to soil following approximately 10 days of growth on agar plates, while subsequent generations were sowed directly on soil. For seedling growth assays on vertical plates, sterilized homozygous seeds were plated in a single row on half-strength Murashige and Skoog media lacking sucrose and antibiotics.

In every case, *Arabidopsis* seeds were cold-treated at 4 ºC for 3 days in the dark prior to their placement in a growth chamber at 22 ºC with a photoperiod of 18 h day/6 h night and 250 µmol/m^2·s light intensity for the remainder of their life cycle. The presence of the AIL7-OE construct was confirmed in transgenic plants by PCR and homozygous lines were identified using segregation analyses. All experiments in which T_1 plants were utilized included empty-vector transformed lines as wild-type controls, while those involving subsequent generations utilized null-segregants as the wild-type control.

Quantitative real-time RT-PCR assessment of *AIL7* transcript levels

Quantitative real-time RT-PCR (qRT-PCR) assays were conducted in triplicate using 1 µl of a 1/50 dilution of each cDNA as template along with SYBR green PCR master mix (Thermo Fisher Scientific, Inc.) in a final volume of 10 µl. Assays were carried out on an ABI 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.) using the *AIL7*-specific primers shown in Table S1. Primers AtPP2AAF1 and AtPP2AAR1 (Singer et al. 2016) were utilized to amplify a 146-nt fragment of the constitutively expressed *PROTEIN PHOSPHATASE 2A SUBUNIT 3* (*PP2AA3*) transcript (Czechowski et al. 2005), which was used as an internal control. Thermal parameters for amplification were as follows: 95 ºC for 2 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Dissociation curves were produced to confirm the presence of a single amplification product in each case. Levels of gene expression were determined using the standard curve method and SDS v2.4 software (Thermo Fisher Scientific, Inc.), with all *AIL7* expression data comprising mean values of biological replicates normalized to those of the *PP2AA3* internal control.

Seed lipid analysis

Arabidopsis seed lipid analysis was carried out as described in our previous study (Singer et al. 2016). In brief, approximately 10 mg dry, mature Arabidopsis seeds (two technical replicates from each plant analyzed) were transmethylated in 3 N methanolic HCl containing 0.1 mg triheptadecanoin (C17:0 TAG in 100 µl chloroform) as an internal standard at 80 ºC for 16 h. The reaction was arrested through the addition of 0.9% (w/v) NaCl and the resulting FA methyl esters (FAMEs) were then extracted twice with hexanes. The FAME extracts were dried under a stream of nitrogen gas and re-suspended in 1 ml iso-octane containing 0.1 mg methyl heneicosanoin (C21:0 methyl ester) as an additional internal standard. Extracted FAMEs were analyzed using an Agilent 6890 Network GC system equipped with a DB-23 capillary column (30 m × 0.25 mm × 0.25 um) and a 5975 inert XL Mass Selective Detector (Agilent Technologies Canada Inc., Mississauga, ON) with the following temperature program: 100 ºC, hold for 4 min, 10 ºC/min to 180 ºC, hold for 5 min, and 10 ºC/min to 230 ºC, hold for 5 min.

Morphological analyses

Weights of T_3 seeds from homozygous AIL7-OE lines and wild-type plants were calculated by weighing small batches of seeds and counting using a FluorChem SP Imager and AlphaEase software (Alpha Innotech Corp., San Leandro, CA). Two to three technical replicates were carried out for 4–6 individual plants (biological replicates) of each line. Seed areas from the same lines were determined using the particle analysis function within ImageJ software (http://imagej.nih.gov/ij). Seedling root growth was assessed by measuring the root lengths of seedlings grown vertically on agar plates at various time points following germination. Experiments were repeated twice.

Scanning electron microscopy

Dry mature T_3 seeds from homozygous AIL7-OE-1 and AIL7-OE-19 lines, along with wild-type plants, were sputter coated with gold/palladium for 1.5 min using a Hummer 6.2 Sputter Coater (Anatech Ltd., Battle Creek, MI). Microscopy was carried out using an FEI scanning electron microscope (model XL30; FEI Company, Hillsboro, OR) operating at 20 kV.

Illumina RNA-Seq

Total RNA from T_3 siliques (14 DAF) of three biological replicates of wild-type and three biological replicates of homozygous AIL7-OE lines (two replicates of AIL7-OE-1 and one replicate of AIL7-OE-19) was processed for RNA-Seq using the Illumina TruSeq Stranded mRNA LT Sample Prep Kit according to the manufacturer's directions (Illumina, San Diego, CA). The quality of the resulting strand-specific libraries were assessed using a BioAnalyzer (Agilent 2100 Bioanalyzer Model G2938C; Agilent Technologies Canada Inc.) and quantities were determined using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Inc.). Libraries were sequenced on an Illumina NextSeq 500 system using

the single-end mode. RNA-Seq reads (75-bp in length) were initially aligned to the Arabidopsis genome using Tophat (Kim et al. 2013), and following alignment, the count of mapped reads from each sample was derived and normalized to reads per kb of exon model per million mapped reads (RPKM). DEGs between wild-type and AIL7-OE lines were identified using the DESeq 1.8.3 package (Nadler et al. 1997) with the raw count data. Raw *P* values were adjusted for multiple testing using a false discovery rate (FDR) (Benjamini and Hochberg 1995), and genes with an FDR of less than 0.05 and fold-changes greater than 2 or less than 0.5 were regarded as DEGs. Two independent AIL7-OE lines were utilized in the analysis as a means of preventing the identification of alterations that may have arisen due to transgene insertion position rather than the transgene itself. GO functional classification of DEGs was achieved using the Plant MetGenMAP program (Joung et al. 2009).

Verification of RNA-Seq results was conducted by carrying out qRT-PCR assays of seven transcripts known to be involved in various lipid metabolic pathways (for primer sequences see Table S1), as well as *AIL7*. Assays were carried out using total RNA derived from 14 DAF siliques from homozygous AIL7-OE-1 and AIL7-OE-19 lines (4–5 biological replicates total), as well as wild-type controls (3 biological replicates). Reactions were carried out as described above. Three technical replicates were used in each case. The correlation coefficient between the $\log_2$ fold-changes in expression derived from qRT-PCR and RNA-Seq was calculated using excel.

In silico analyses of predicted TF-binding sites

The Plant Cistrome Database (http://neomorph.salk.edu/PlantCistromeDB) was utilized for the identification of putative TF target genes based on their predicted DNA-binding motifs (O'Malley et al. 2016).

Results

Confirmation of *AIL7* over-expression in AIL7-OE lines

Arabidopsis seed-specific *AIL7* over-expression lines were generated through the introduction of a cassette in which the seed-specific *napin* promoter was used to drive the expression of the *Arabidopsis AIL7* coding sequence (Fig. 1A). This promoter is known to elicit particularly high levels of expression of associated transgenes during the heart to torpedo stage of embryo development, and lacks activity in vegetative tissues, such as leaves and roots (Ellerström et al. 1996; Stålberg et al. 1993). To confirm the up-regulation of *AIL7* expression in our transgenic lines, we analyzed developing T_2 siliques containing T_3 seeds (14 DAF) from the two independent over-expression lines utilized throughout this study (AIL7-OE-1 and AIL7-OE-19), as well as wild type, using qRT-PCR. AIL7-OE-1 lines exhibited a 33-fold increase in expression compared to wild type, while AIL7-OE-19 lines exhibited a 19-fold increase in expression compared to wild type (Fig. 1B). These results confirm the over-expression of the *AIL7* transcript in these lines.

To assess *AIL7* expression across the mid-stages of seed development when seed oil begins to accumulate (Baud et al. 2008), we also tested developing siliques at various DAF during the mid-stage of seed development (Figs. S1A and S1B). Total RNA was extracted from homozygous T_3 AIL7-OE-1 and wild-type siliques 10, 11, 12, 13, 14 and 15 DAF for qRT-PCR assays. In wild-type plants, we observed a gradual increase in expression of *AIL7* from 11 DAF until 14 DAF, at which point expression leveled off (Fig. S1A). In AIL7-OE-1 siliques, expression of *AIL7* also increased between 11 and 14 DAF, after which point expression began to drop (Fig. S1B). At every time point analyzed (10–15 DAF), expression of *AIL7* was substantially enhanced in AIL7-OE-1 lines compared to wild-type. This enhancement due to the *AIL7* over-expression remained relatively constant between 11 and 14 DAF; where we observed increases of 25- and 31-fold compared to wild-type; which is reflective of the value seen in AIL7-OE-1 T_2 siliques at 14 DAF. However, the difference between AIL7-OE-1 and wild-type lines began to decrease at 15 DAF (11-fold increase in expression compared to wild-type). Therefore, due to the peak in *AIL7* expression in wild-type lines and the very high fold-change seen in the expression of this gene between AIL7-OE and wild-type lines at 14 DAF, we chose this time point for subsequent RNA-Seq analysis.

AIL7-OE lines exhibit alterations in fatty acid composition and seed oil content

Compositional analysis of seed oil from AIL7-OE and wild-type lines demonstrated very substantial alterations in the proportions of FAs, the pattern of which was identical over both generations tested and multiple independent transgenic lines (Figs. 2A and S2A). With the exception of C16:1, the proportions of all FAs within the seed oil were affected consistently to a significant degree. In T_3 seeds derived from two independent homozygous AIL7-OE lines, average relative increases of 38.48% ± 1.23 SE (C16:0), 33.02% ± 0.35 (C18:1) and 39.93% ± 6.27 (C18:2) were observed compared to wild-type, whereas average relative decreases of 8.76% ± 0.35 (C18:0), 52.55% ± 1.72 (C18:3), 39.41% ± 1.28 (C20:0), 37.06% ± 2.59 (C20:1), 47.00% ± 1.23 (C20:2), 79.42% ± 1.06 (C20:3), 21.29% ± 0.23 (C22:0) and 52.39% ± 2.82 (C22:1) were observed compared to wild-type (Fig. 2A).

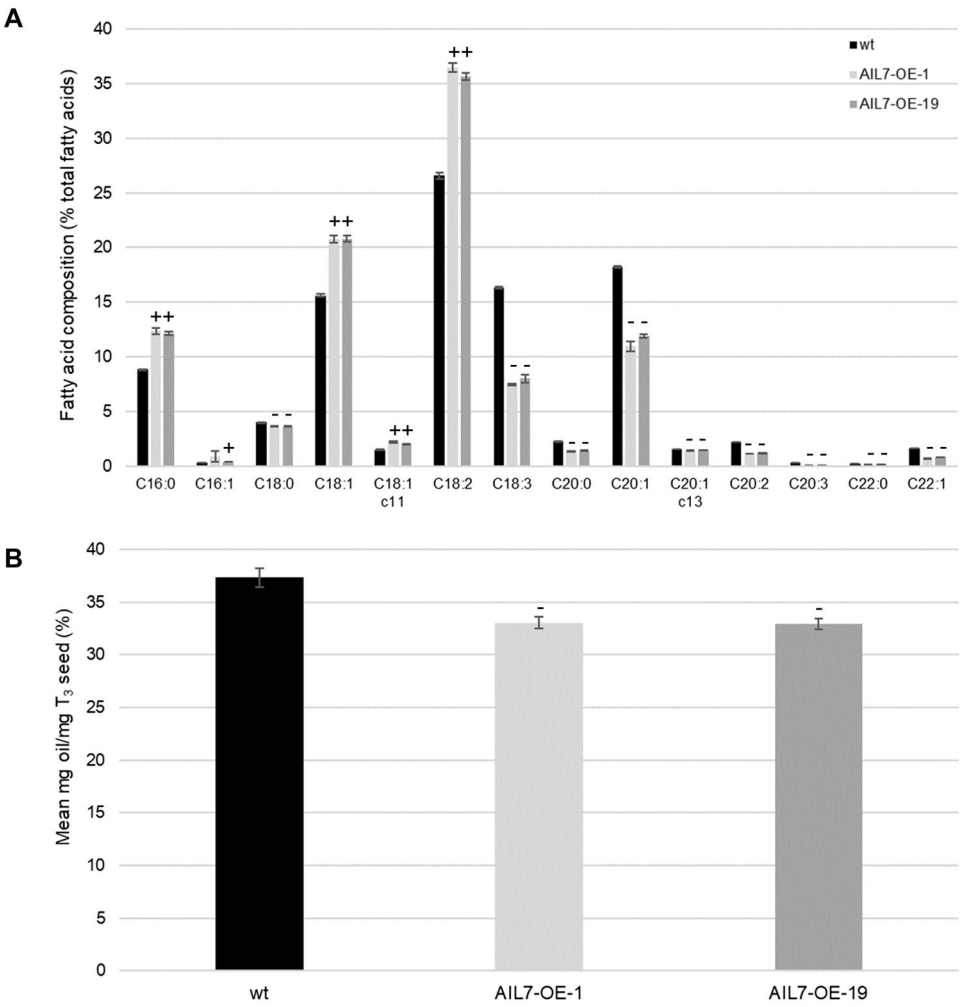

Fig. 2 Fatty acid composition and oil content of homozygous T_3 AIL7-OE and wild-type seeds. **A** Mean fatty acid composition of oil from homozygous T_3 AIL7-OE seeds. Blocks indicate mean values of wild-type ($n=6$) and two independent AIL7-OE lines (AIL7-OE-1 [$n=6$] and AIL7-OE-19 [$n=5$]). **B** Mean seed oil content of T_3 seeds. Blocks represent mean values from wild-type ($n=6$) and two independent homozygous AIL7-OE lines (AIL7-OE-1 [$n=6$] and AIL7-OE-19 [$n=5$]). Two technical replicates were carried out for each line analyzed. Bars denote standard errors. Very significant increases and decreases compared to wild-type (as measured by 2-tailed Student's *t* test assuming unequal variance) are indicated by + and − ($P \leq 0.01$). *wt* wild-type

Seed oil content was also affected in AIL7-OE lines, with significant reductions apparent in the seeds of both generations tested (Figs. 2B and S2B). Indeed, in homozygous T_3 seeds, relative decreases of 11.52% (AIL7-OE-1) and 11.80% (AIL7-OE-19) were observed compared to wild-type (Fig. 2B).

Seed-specific over-expression of *AIL7* results in changes to seed morphology

Both light and scanning electron microscopy revealed that transgenic AIL7-OE seeds exhibited morphological changes compared to wild-type (Fig. 3). Light microscopy demonstrated that seeds from the transgenic lines appeared twisted or bent in shape, and were not elliptical as were wild-type seeds (Fig. 3A). SEM micrographs confirmed these findings, displaying transgenic seeds that bore ridges that twisted around the seed (Fig. 3B). While significant alterations in seed weights of AIL7-OE lines compared to wild-type plants were not evident (Fig. 3C), small increases in seed area were observed in AIL7-OE plants, although this difference was only significant in the AIL7-OE-1, but not the AIL7-OE-19, homozygous line (Fig. 3D).

Seed-specific over-expression of *AIL7* leads to a reduction in seedling growth

To establish whether there were any carry-over effects in the next generation of AIL7-OE lines, we further assessed the morphology of transgenic and wild-type seedlings. As was the case for wild-type seedlings, homozygous T_3 AIL7-OE seedlings grown vertically on solid medium germinated within 24 h; however, once germinated, transgenic seedlings exhibited a significant decrease in their rates of growth compared to wild-type (Fig. 4). At 9 days post germination, seedling root lengths were reduced on average 60.1% (AIL7-OE-1) and 47.1% (AIL7-OE-19) compared to wild type (Fig. 4B). AIL7-OE seedlings grown on soil also appeared to exhibit reduced size of rosettes (Fig. 4C) and shorter root lengths but bolted and reached maturity at similar time points as wild-type plants (data not shown). In addition, mean overall seed yield of the

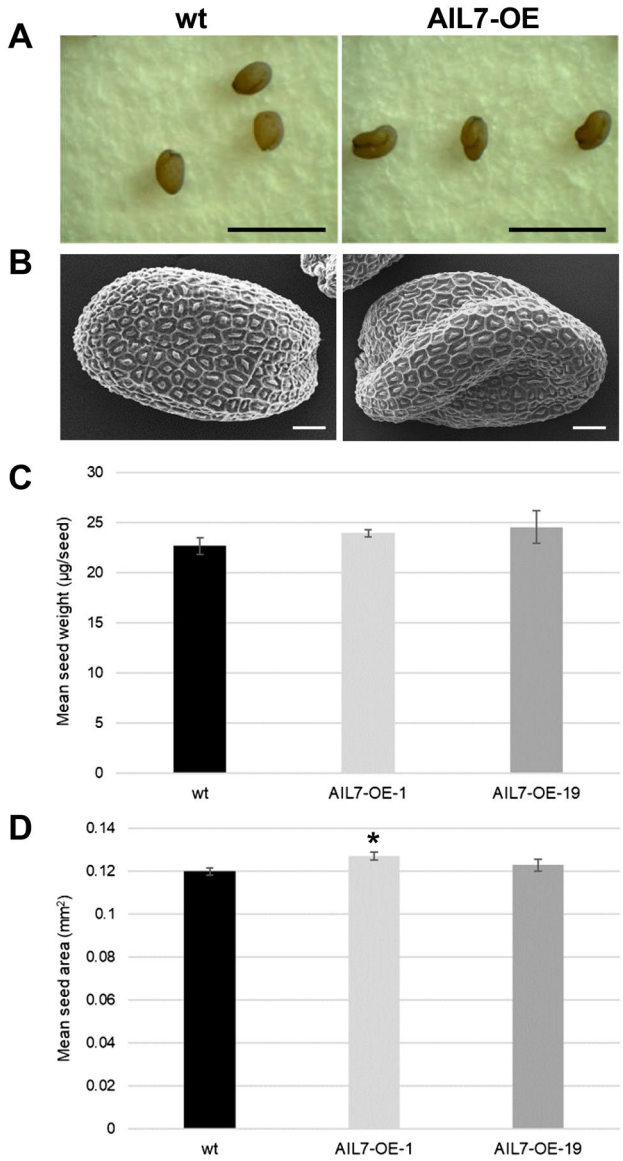

Fig. 3 Seed morphology of AIL7-OE lines. **A** Representative light microscopic images of wild-type and homozygous T_3 AIL7-OE mature seeds. Scale bars = 1 mm. **B** Representative SEM images of wild-type and homozygous T_3 AIL7-OE seeds. Scale bars = 100 µm. **C** Mean mature seed weights from wild-type (from 6 individual plants), as well as homozygous T_3 AIL7-OE-1 (from 6 individual plants) and AIL7-OE-19 (from 4 individual plants) lines. Two to three replicate seed batches were used for each measurement. **D** Mean mature seed areas of wild-type ($n = 61$ from 3 individual plants), as well as homozygous T_3 AIL7-OE-1 ($n = 88$ from 3 individual plants) and AIL7-OE-19 ($n = 53$ from 3 individual plants) lines. Asterisks denote significant differences from wild type as determined by 2-tailed Student's *t* tests assuming unequal variance, $P \leq 0.01$

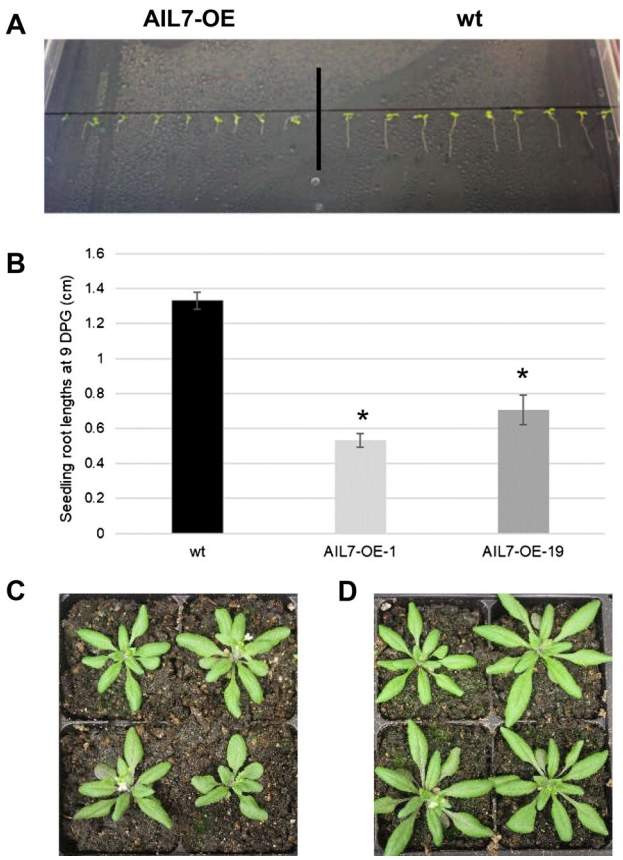

Fig. 4 Growth of homozygous T_3 AIL7-OE and wild-type seedlings. **A** Seedlings grown vertically on solid medium were photographed 9 days post-germination and are representative of two independent experiments. AIL7-OE seedlings are shown to the left of the black vertical line, while wild-type seedlings are present to the right**. B** Root lengths of wild-type ($n = 35$), AIL7-OE-1 ($n = 20$), and AIL7-OE-19 ($n = 16$) seedlings grown vertically on solid medium 9 days post-germination. Blocks denote mean values and bars indicate standard errors. Asterisks denote means that are significantly different from wild-type as determined by 2-sided Student's *t*-tests assuming unequal variance ($P \leq 0.01$). **C and D** Representative soil-grown AIL7-OE **(C)** and wild-type **(D)** lines 24 days post germination. *DPG* days post-germination, *wt* wild type

two homozygous AIL7-OE T_3 lines combined (212.43 mg/plant ± 6.75 SE; $n = 64$) was also slightly lower than that of wild type (234.36 mg/plant ± 8.20 SE; $n = 34$), a difference that was significant at $P \leq 0.05$.

Differential expression in AIL7-OE vs. wild-type silique transcriptomes

To determine how the over-expression of *AIL7* affects the transcriptome in developing Arabidopsis seeds, we carried out RNA-Seq analysis using RNA derived from developing siliques (14 DAF) of homozygous AIL7-OE lines and wild-type plants (Table S2). We identified a total of 27,202 expressed sequences in our transgenic and wild-type lines with 1682 of these genes displaying significantly increased transcript abundance up-regulated Differentially Expressed Genes (DEGs) and 378 exhibiting significantly decreased transcript abundance (down-regulated DEGs) in transgenic lines compared to wild type (Supplemental Data S1).

Gene ontology classification revealed differential expression of genes related to many biological processes, molecular functions and cellular compartments

Gene ontology (GO) classification of the resulting DEGs indicated that they fell within several biological process categories (Fig. S3). For instance, many DEGs were involved in photosynthesis (GO:0015979; 28 genes up-regulated), response to stress (GO:0006950; 137 genes up-regulated and 27 genes down-regulated), embryonic development (GO:0009790; 19 genes up-regulated and 5 genes down-regulated) and flower development (GO:0009908; 17 genes up-regulated and 8 genes down-regulated), for example. Furthermore, 45 genes were up-regulated and 20 genes were down-regulated within the lipid metabolic process (GO:0006629; Table S3).

DEGs were also classified based on their molecular function (Fig. S4), as well as their cellular component (Fig. S5). Within the molecular function category, groups with a high abundance of DEGs included those relating to protein binding (GO:0005515; 191 up-regulated and 22 down-regulated), hydrolase activity (GO:0016786; 180 up-regulated and 40 down-regulated), kinase activity (GO:0016301; 129 up-regulated and 10 down-regulated), catalytic activity (GO:0003824; 143 up-regulated and 54 down-regulated), nucleotide-binding (GO:0000166; 129 up-regulated and 11 down-regulated) and DNA bindng (GO0003677; 104 up-regulated and 22 down-regulated). Other interesting groups included TF activity (GO:0030528; 91 up-regulated and 45 down-regulated) and lipid binding (GO:0008289; 13 up-regulated and 3 down-regulated). Within the cellular component category, a number of DEGs were classified within the nucleus (GO:0005634; 131 up-regulated and 28 down-regulated), plastid (GO:0009536; 196 up-regulated and 19 down-regulated) and thylakoid (GO:0009579; 55 up-regulated and 1 down-regulated). Thirteen and four genes encoding proteins localized to the ER were also differentially up-regulated or down-regulated, respectively, between AIL7-OE and wild-type developing siliques (GO:0005783).

AIL7 over-expression affects the expression of genes involved in fatty acid biosynthesis and triacylglycerol accumulation

At least 17 DEGs with possible involvement in seed FA or TAG biosynthesis were identified in developing siliques from our AIL7-OE lines compared to wild-type plants (Table 1; Fig. 5). Up-regulated DEGs included an *ACYL CARRIER PROTEIN 5* (*ACP5*; AT5G27200), *LONG-CHAIN ACYL-COA SYNTHETASE 9* (*LACS9*; AT1G77590), *3-KETOACYL-ACYL CARRIER PROTEIN SYNTHASE I* (*KASI*; AT5G46290), and *LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE 5* (*LPAT5*; AT3G18850) for example. Down-regulated DEGs included *3-KETOACYL-COA SYNTHASE 5* (AT1G25450), *KCS7* (AT1G71160), *FATTY ACID ELONGATION 1* (*FAE1*; AT4G34520) and *B-KETOACYL-COA REDUCTASE 2* (*KCR2*; AT1G24470). In addition, although it did not meet our chosen threshold ratio of 0.5, *FATTY ACID DESATURASE 3* (*FAD3;* AT2G29980), which is essential for the desaturation of C18:2 to C18:3, was also significantly down-regulated by a ratio of 0.52 in AIL7-OE lines compared to wild type. Similarly, *PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE 2* (*PDAT2*; AT3G44830) and *OLEOSIN 1* (*OLEO1*; AT4G25140) were also significantly down-regulated with a ratio of 0.59 and 0.76 in AIL7-OE lines. Conversely, *VIVIPAROUS1/ABI3-LIKE1* (*VAL1*; AT2G30470) was significantly up-regulated in AIL7-OE lines with a ratio of 1.67. A number of genes with possible functions related to FA or TAG breakdown were also differentially expressed in AIL7-OE lines compared to wild type, including *SUGAR-DEPENDENT1-LIKE* (*SDP1L*; AT3G57140; Table 2), which plays a known role in catalyzing TAG hydrolysis in Arabidopsis seeds (Kelly et al. 2011). The expression levels of many other genes encoding TFs, enzymes and biologically active proteins known to function in FA and TAG biosynthesis in developing seeds were not significantly affected by the over-expression of *AIL7* (Table S4).

AIL7 over-expression leads to the up-regulation of genes involved in starch metabolism

Fourteen genes involved in starch biosynthesis and degradation were also differentially expressed in developing siliques from AIL7-OE lines compared to wild-type, with the genes being significantly up-regulated in every case (Table S5). These included genes involved in starch biosynthesis, such as *ADP-GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 2* (*APS2*; AT1G05610) and *ADP-GLUCOSE PYROPHOSPHORYLASE LARGE SUBUNIT 2* and *4* (*APL2* and *APL4*; AT1G27680 and AT2G21590). While up-regulation of *STARCH SYNTHASE 3* (*SS3*; AT1G11720) did not meet our threshold ratio of 2, its 1.8-fold up-regulation in AIL7-OE lines compared to wild type was significant. Up-regulation was also observed in genes involved in starch branching, including *STARCH BRANCHING ENZYME 2.1* and *2.2* (*SBE2.1* and *SBE2.2*; AT2G36390 and AT5G03650), and de-branching (*ISOAMYLASE 1* [*ISA1*]; AT2G39930). Finally, up-regulation was also apparent in various genes involved in starch degradation, including *α-GLUCAN PHOSPHORYLASE 2* (*PHS2*; AT3G46970), *β-AMYLASE 2* (*BAM2*; AT4G00490), *DISPROPORTIONATING ENZYME 1* and *2* (*DPE1* and *DPE2*; AT5G64860 and AT2G40840), *STARCH EXCESS 1* and *4* (*SEX1* and *SEX4*; AT1G10760 and AT3G52180), and *LIKE SEX4 2* (*LSF2*; AT3G10940).

Table 1 Effect of *AIL7* over-expression on the expression of a selection of genes with known or putative functions in seed fatty acid and triacylglycerol biosynthesis

Gene ID			Ratio	*P* value	Description
Transcriptional regulators					
AT2G30470	1.67			1.87e–2	VAL1 (VIVIPAROUS1/ABI3-LIKE1)
FA biosynthesis/modification					
AT1G25450		0.09		5.08e–6	KCS5 (3-ketoacyl-CoA synthase 5)
AT1G71160		0.09		3.08e–2	KCS7 (3-ketoacyl-CoA synthase 7)
AT4G34520		0.16		8.91e–6	FAE1 (FATTY ACID ELONGASE 1)
AT1G24470		0.32		1.70e–3	KCR2 (B-KETOACYL-COA REDUCTASE 2)
AT4G16210		0.48		1.07e–7	Enoyl-Coa hydratase/isomerase family protein
AT2G29980		0.52		4.44e–4	FAD3 (FATTY ACID DESATURASE 3)
AT5G46290		2.42		5.59e–4	KAS I (3-ketoacyl-acyl-carrier protein synthase I)
AT1G36180		2.43		7.74e–7	ACC2 (ACETYL-COA CARBOXYLASE 2)
AT4G00520		2.51		7.64e–5	Acyl-CoA thioesterase family protein
AT5G27200		5.54		1.65e–5	ACP5 (ACYL CARRIER PROTEIN 5)
AT1G77590		5.63		1.41e–7	LACS9 (LONG-CHAIN ACYL-COA SYNTHETASE 9)
TAG biosynthesis and storage					
AT3G44830			0.59	4.3e–2	PDAT2 (PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE 2)
AT4G25140			0.76	4.2e–2	OLEO1 (OLEOSIN 1)
AT4G37740			2.38	3.07e–4	GRF2 (GROWTH-REGULATING FACTOR 2-LIKE)
AT3G18850			6.23	5.64e–11	LPAT5 (LYSOPHOSPHATIDYL ACYLTRANSFERASE 5)
AT3G11325			12.3	2.79e–2	Putative acyltransferase

Raw *P* values derived from comparative RNA-Seq analysis were adjusted for multiple testing using a false discovery rate (FDR), and genes that are up-regulated or down-regulated in AIL7-OE lines compared to wild-type plants with an FDR of less than 0.05 are listed here. Ratios indicate relative expression levels in AIL7-OE lines compared to wild-type plants

Effect of AIL7 over-expression on the expression of genes involved in processes other than storage compound accumulation

In addition to its effect on the expression of genes involved in TAG and starch accumulation, the over-expression of *AIL7* also significantly affected the expression of five of the seven other *AIL/PLT* subfamily genes, including *BBM* and *PLT2*, which were up-regulated, and *AIL1*, *AIL5* and *AIL6*, which were down-regulated (Table S6). A number of other genes falling within meristem-related GO biological process categories were also differentially expressed in developing siliques from AIL7-OE lines (Table S7), with the majority being up-regulated. However, genes encoding major meristem regulators, such as *CLAVATA 1*, *2* and *3* (*CLV1*, *2* and *3*; AT1G75820, AT1G65380, AT2G27250), *WUSCHEL* (*WUS*; AT2G17950) *SHOOTMERISTEMLESS* (*STM*; AT1G62360) and *CUP SHAPED COTYLEDON 1* and *2* (*CUC1* and *2*; AT3G15170 and AT5G53950), were not altered at the transcriptional level in AIL7-OE lines compared to wild type.

Interestingly, a large proportion of genes with functions within the photosynthetic process, and especially the light reactions, were also up-regulated in developing siliques from AIL7-OE lines compared to wild type, while no genes with roles within this pathway were down-regulated (Table S8). In terms of genes functioning within the response to stress GO cellular process category, 164 genes were differentially expressed (Table S9), including 27 that were significantly down-regulated and 137 that were up-regulated. Genes within anatomical structure morphogenesis (Table S10) and embryonic development (Table S11) GO biological process categories were also differentially regulated in AIL7-OE lines compared to wild type.

Verification of RNA-Seq data

To validate our RNA-Seq expression profile data, we performed quantitative real-time RT-PCR on seven genes encoding enzymes involved in lipid metabolic pathways, including *AIL7*, *FAE1*, *FAD3*, *ABCG12, ACP5*, *GPAT1*, *LACS9* and *KASI*, which were found to exhibit significant alterations in their expression levels in developing siliques from transgenic AIL7-OE lines compared to wild-type in the RNA-Seq experiment. In every case, $\log_2$ fold-changes in expression between transgenic lines and wild-type plants corresponded well with our RNA-Seq results (Fig. 6), yielding a correlation coefficient of 0.981173.

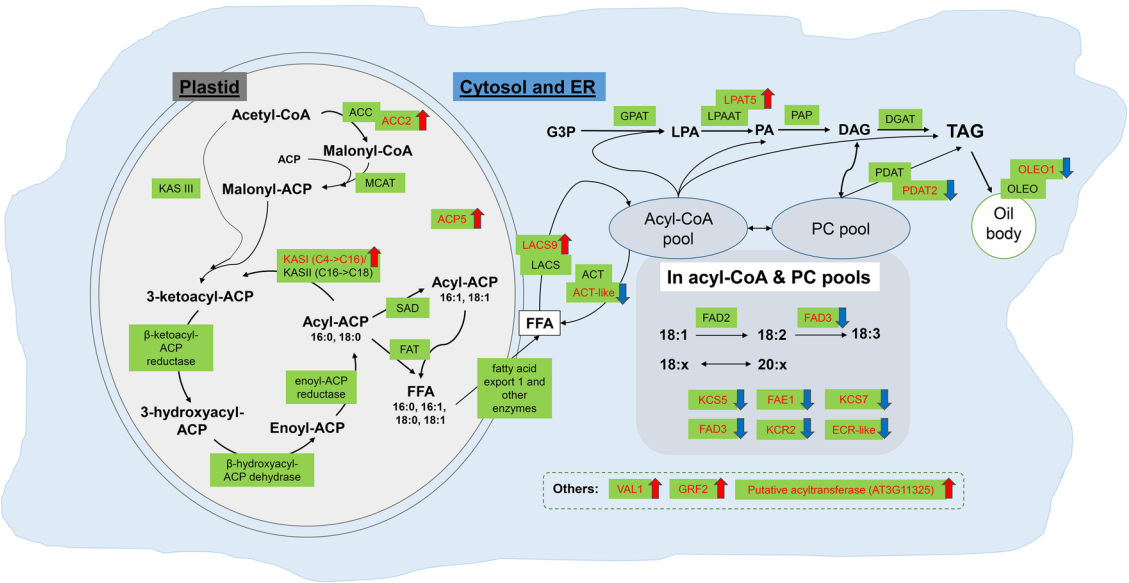

Fig. 5 Simplified diagrammatic representation of fatty acid and triacylglycerol biosynthesis in *Arabidopsis* seeds. A selection of genes with known or putative functions in seed fatty acid and triacylglycerol biosynthesis are displayed, with those transcriptionally affected by *AIL7* over-expression labeled with red or blue arrows to represent up- or down-regulation, respectively. Enzymes/genes are displayed within green rectangles. *ACC* acetyl-CoA carboxylase, *ACP* acyl-carrier protein, *ACT* acyl-CoA thioesterase, *DAG* diacylglycerol, *DGAT* diacylglycerol acyltransferase, *ECR* enoyl-CoA reductase, *ER* endoplasmic reticulum, *FAD* fatty acid desaturase, *FAE* fatty acid elongase, *FAT* acyl-ACP thioesterase, *FFA* free fatty acid, *G3P sn*-glycerol-3-phosphate, *GPAT sn*-glycerol-3-phosphate acyltransferase, *GRF2* growth-regulating factor 2-like, *KAS* ketoacyl-ACP synthase, *KCR* ketoacyl-CoA reductase, *KCS* ketoacyl-CoA synthase, *LACS* long-chain acyl-CoA synthase, *LPA* lysophosphatidic acid, *LPAAT* acyl-CoA:lysophosphatidic acid acyltransferase, *MCAT* malonyl-CoA: ACP acyltransferase, *OLEO* Oleosin, *PA* phosphatidic acid, *PAP* phosphatidic acid phosphatase, *PC* phosphatidylcholine, *PDAT* phospholipid:diacyglycerol acyltransferase, *SAD* stearoyl-ACP desaturase, *TAG* triacylglycerol, *VAL1* VIVIPAROUS1/ABI3-LIKE1. This model was developed based on the transcriptome data garnered in the current study, as well as information from Chen et al. (2015) and Tian et al. (2019)

Predicted direct targets of AIL7 and other TFs in the seed lipid regulatory network

Comparison of predicted AIL7 target genes derived from the Plant Cistrome Database, which were established using DNA affinity purification sequencing (DAP-Seq; O'Malley et al. 2016), with DEGs revealed through our RNA-Seq analysis led to the identification of 51 genes that are possible direct targets of AIL7 in the seeds of our over-expression lines (Table S12). All but three of these DEGs were up-regulated in AIL7-OE lines compared to wild-type plants. Interestingly, *AIL7* was also listed as a putative target gene for the AGL15 (AT5G13790) and ABI5 (AT2G36270) TFs in the Plant Cistrome Database.

Discussion

The biosynthesis and accumulation of FAs and TAG in plants are extremely complex processes involving an incredibly large number of genes, which are regulated at the epigenetic, transcriptional and post-transcriptional levels (e.g., Kong et al. 2019; Kumar et al. 2020). Although enormous efforts have been undertaken to unravel these regulatory pathways in full, several gaps remain in our overall understanding of them. The LAFL TFs have been identified as master regulators of seed maturation and seed oil accumulation, affecting the expression of multiple other genes encoding TFs, enzymes and biologically active proteins involved in lipid biosynthesis (Fatihi et al. 2016). However, this list of TFs is by no means exhaustive, and other, as of yet unidentified TFs likely also contribute to this process. As such, we sought to examine the function of *Arabidopsis AIL7*, which is expressed at relatively high levels during the maturation phase of seed development when storage oil begins to accumulate (Baud et al. 2008) and encodes a TF that has yet to be characterized in this context (Le et al. 2010), by investigating the effects of its over-expression in *Arabidopsis* seeds and assessing global transcriptomic changes in these lines compared to wild type.

Quantitative real-time RT-PCR of wild-type plants indicated that *AIL7* expression increased during the mid-stages of *Arabidopsis* seed development (at which time storage oil reserves are accumulating) until 14 DAF, after which time expression began to level off (Fig. S1A), which corresponds well with a previous expression analysis of this

Table 2 Effect of *AIL7* over-expression on the expression of genes with known or putative functions in fatty acid or triacylglycerol breakdown

Gene ID	Ratio	*P* value	Description
AT1G54000	0.11	5.94e–3	GDSL-like lipase/acyl-hydrolase superfamily protein
AT2G42990	0.17	3.85e–2	GDSL-like lipase/acyl-hydrolase superfamily protein
AT1G54010	0.24	2.91e–3	GDSL-like lipase/acyl-hydrolase superfamily protein
AT3G05180	0.26	2.00e–2	GDSL-like lipase/acyl-hydrolase superfamily protein
AT5G03610	0.34	2.05e–11	GDSL-like lipase/acyl-hydrolase superfamily protein
AT1G51440	0.39	1.52e–2	DAD1-like lipase 2
AT5G22810	0.41	4.13e–3	GDSL-like lipase/acyl-hydrolase superfamily protein
AT1G28610	0.45	1.03e–2	GDSL-like lipase/acyl-hydrolase superfamily protein
AT4G16210	0.48	1.07e–7	Enoyl-CoA hydratase/isomerase family protein
AT3G07400	2.19	2.18e–5	Lipase class 3 family protein
AT5G14450	2.24	1.86e–4	GDSL-motif lipase/acyl-hydrolase superfamily protein
AT1G09390	2.26	1.97e–2	GDSL-motif lipase/acyl-hydrolase superfamily protein
AT4G01130	2.44	3.29e–2	GDSL-like lipase/acyl-hydrolase superfamily protein
AT1G71250	2.83	3.94e–3	GDSL-motif lipase/acyl-hydrolase superfamily protein
AT1G06800	2.87	4.51e–6	DAD1-like lipase 4
AT3G57140	3.1	1.24e–5	SDP1L (SUGAR-DEPENDENT 1-LIKE)
AT1G75900	3.45	3.63e–4	GDSL-motif lipase/acyl-hydrolase superfamily protein
AT5G45670	3.59	1.62e–3	GDSL-motif lipase/acyl-hydrolase superfamily protein
AT5G03820	4.33	5.98e–3	GDSL-motif lipase/acyl-hydrolase superfamily protein
AT5G24210	4.69	1.48e–2	Lipase class 3 family protein
AT1G71120	6.13	1.39e–10	GLIP6 (GDSL-motif lipase/hydrolase 6)
AT4G10950	80.6	2.62e–8	GDSL-type esterase/lipase 77

Raw *P* values derived from comparative RNA-Seq analysis were adjusted for multiple testing using a false discovery rate (FDR), and genes with an FDR of less than 0.05 are listed here. Ratios indicate relative expression levels in AIL7-OE lines compared to wild-type plants

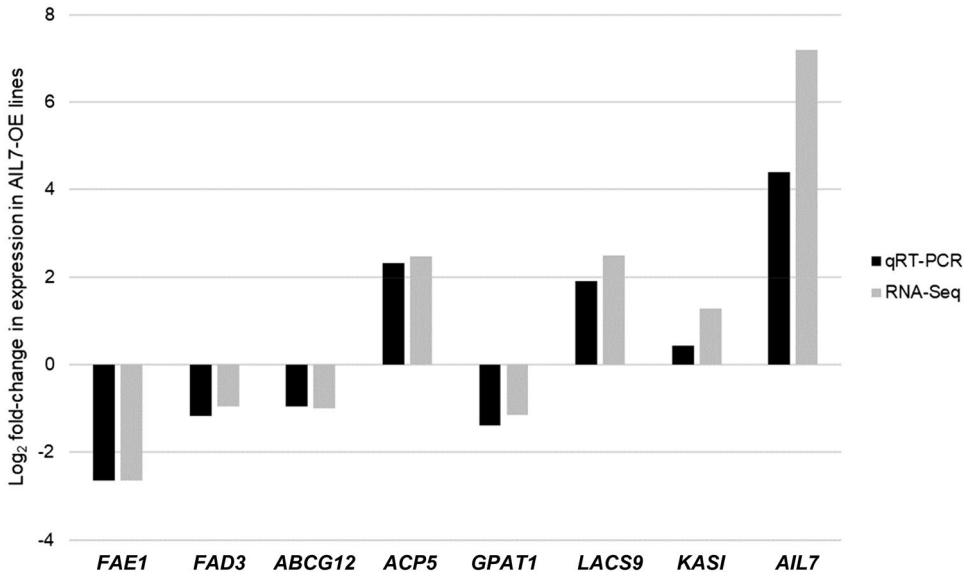

Fig. 6 qRT-PCR validation of RNA-Seq results. qRT-PCR was carried out on RNA derived from the developing siliques (14 DAF) of AIL7-OE (4–5 biological replicates) and wild-type (3 biological replicates) lines, using the internal control, *PP2AA3,* as a reference. Three technical replicates were carried out in each case. Blocks represent $\log_2$ fold-changes in expression derived from qRT-PCR and RNA-Seq data, respectively. *ABCG12* ABC-2 type transporter family protein, *ACP5* acyl-carrier protein 5, *AIL7* AINTEGUMENTA-LIKE 7, *FAD3* fatty acid desaturase 3, *FAE1* fatty acid elongase 1, *GPAT1* glycerol-3-phosphate acyltransferase 1, *KASI* 3-ketoyacyl-acyl-carrier protein synthase I, *LACS9* long-chain acyl-CoA synthetase 9

gene in developing *Arabidopsis* seeds (Le et al. 2010). Over-expression of *AIL7* in *Arabidopsis* seeds also resulted in high levels of up-regulation during the maturation phase of seed development, peaking in siliques harvested 14 DAF (Figs. 1B and S1B), which derives from the spatiotemporal activity of the *napin* promoter used to drive *AIL7* expression.

Intriguingly, in AIL7-OE lines, both T_2 and T_3 seeds exhibited consistent and significant alterations in the FA profile of their seed oil (Figs. 2A and S2A). Specifically, the proportions of C16:1, C18:1 and C18:2, increased significantly in transgenic lines. Conversely, the proportion of C18:0 and C18:3, as well as all very-long-chain FAs (VLCFAs) with carbon chain lengths above 20, decreased significantly in AIL7-OE lines compared to wild type. These alterations in FA composition correlated well with changes in the expression of particular genes that were observed in our RNA-Seq analysis (Supplemental Data S1; Fig. 5). For instance, an increased amount of C16:0 in seed oil could have resulted, at least in part, from the up-regulation of *KASI* (Table 1; Fig. 6), which utilizes C4:0- to C14:0-ACPs as substrate in a condensation reaction with malonyl-ACP, resulting in the production of C6:0- to C16:0-ACPs (Wu and Xue 2010). Similarly, increased levels of C18:2 and decreased proportions of C18:3 could be explained by down-regulation of *FAD3*, which functions to catalyze the desaturation of C18:2 to produce C18:3 [Table 1; Fig. 6; (Lemieux et al. 1990)]. Furthermore, the reduction in the proportion of FAs with chain lengths longer than C18 likely stemmed from a significant decrease in the expression of genes encoding components of the FAE complex, including the β-ketoacyl-CoA synthases *FAE1*, which encodes a condensing enzyme that is responsible for the production of C20 and C22 FAs in seeds (James et al. 1995), and *KCS7*, which has yet to be functionally characterized in seeds (Table 1). Given that *fae1* mutants display increased accumulation of C16 and C18 FAs concomitant to reductions in VLCFAs, reduction in the expression of this gene could also contribute to the overall increase in C16:0, C18:1 and C18:2 observed in AIL7-OE lines (James et al. 1995). Similarly, a significant increase in the expression of *LACS9*, which encodes a plastidial long-chain acyl-CoA synthetase that is involved in the activation of free FA to acyl-CoA and displays substrate preferences for C16:0, C18:0, C18:1 and C18:2 (Shockey et al. 2002), could also have been a factor in the alterations in fatty acid composition observed in the AIL7-OE lines (Table 1; Fig. 6).

The increases in the proportions of C18:1 and C18:2 combined with the decrease in the proportion of C18:3 for the two AIL7-OE lines suggest that the resulting oils would be more stable to oxidation than oil from wild-type plants. Previous studies have clearly demonstrated that vegetable oils containing lower proportions of C18:3 are more stable under frying conditions (Eskin et al. 1989). Indeed, reducing 18:3 content to increase oxidative stability is a major goal of many Brassica oilseed breeding programs (e.g., Rahman et al. 2013; Singer et al. 2014). Given that the penalty

on seed oil content is relatively low (Fig. 2B), *AIL7* overexpression might be a useful molecular tool for decreasing the C18:3 content of seed oil from Brassica oilseed species to generate an oil with increased oxidative stability.

In addition to the clear shift in FA composition in AIL7-OE seed oil, we also observed a slight but significant reduction in total seed oil content compared to wild-type plants in both the T_2 and T_3 generations (Figs. 2B and S2B). While such an effect may have been elicited in a variety of distinct manners, the differential expression of genes with functions in TAG hydrolysis, the generation of oil bodies and starch biosynthesis were particularly conspicuous as possible contributors. In the context of oilseeds, the breakdown of storage TAG occurs primarily during seedling establishment to provide a source of energy prior to the establishment of photosynthetic competence. Oil breakdown is initiated through the enzymatic action of lipases that catalyze the hydrolysis of TAG to release glycerol and free FAs, which are then transferred to the glyoxysome where they are activated to acyl-CoAs and subsequently catabolized through β-oxidation (Eastmond 2006). Our RNA-Seq results demonstrated that genes encoding several enzymes with possible functions in TAG breakdown were differentially expressed in developing siliques from AIL7-OE lines compared to wild type (Table 2). Perhaps the most noteworthy is the significant up-regulation of *SDP1L*, which encodes a TAG lipase that has been shown previously to play a major role in oil breakdown following seed germination in Arabidopsis (Kelly et al. 2011) and could plausibly have a negative impact on seed oil accumulation. While a small number of the other genes listed have been reported to be involved in fatty acid degradation and/or lipid metabolism (Chen et al. 2012), the biological functions of GDSL proteins in particular remain poorly understood. As such, future studies involving the functional characterization of such genes in lipid catabolism would provide additional knowledge in this area.

In seeds, TAG is stored in specialized oil bodies that are associated with various proteins, with oleosins making up the majority of these (Huang 2018). It has been shown previously that down-regulation/mutation of the major oleosin, *OLEO1*, in *Arabidopsis* led to a substantial reduction in seed TAG, along with a simultaneous increase in storage protein content (Siloto et al. 2006). Therefore, it is feasible that the significant down-regulation of *OLEO1* in AIL7-OE lines compared to wild type may have also played a role in the observed decrease in seed oil content (Table 1).

A trade-off in the accumulation of distinct seed storage compounds (e.g., oil, protein and starch), whereby an increase in the accumulation of one type of compound is associated with a decrease in another, is a fairly common occurrence in plants due to the fact that carbon supplies tend to be limited during seed maturation and the distinct biosynthetic pathways are in competition for resources (e.g., Lin et al. 2006; Wang et al. 2019; Zhang et al. 2010). While starch is an important seed storage product in many cereal grains, it only accumulates transiently during oilseed development, with oil and storage proteins predominantly stored in mature seeds (Mansfield and Briarty 1992). Interestingly, 14 genes involved in starch metabolism were differentially expressed in developing siliques from AIL7-OE lines, with every one of them being significantly up-regulated (Table S5). This included three genes encoding various subunits of ADP-glucose pyrophosphorylase, which catalyzes the first and rate-limiting step of starch biosynthesis (Cross et al. 2004). These findings hint at the possibility that reduced seed oil content in AIL7-OE lines could be linked to an increase in transitory starch. Such a phenomenon has been observed in Arabidopsis *wri1* mutants, where a maximal augmentation in seed starch content was noted 9 days after flowering, with levels dissipating to those seen in wild-type plants by maturity (Focks and Benning 1998). Although starch content was not assessed in AIL7-OE transgenic lines, the fact that we also detected the up-regulation of several genes involved in starch degradation (Table S5) suggests that a similar decline in starch enhancement could be evident. However, further characterization will be required to gain insight into the precise interactions between starch and lipid metabolic pathways in AIL7-OE transgenic plants in the future.

It is not known whether the effects of *AIL7* over-expression on FA biosynthesis and lipid accumulation in seeds result from direct or indirect transcriptional regulation of downstream genes involved in this process. However, a search of putative AIL7 gene targets in the Plant Cistrome Database indicated that although it may directly regulate a number of genes encoding enzymes that were differentially expressed in developing siliques from AIL7-OE lines, the only potential direct target identified in the context of seed storage compounds was the starch biosynthetic gene *SBE2.1* (Table S12). While the list of target genes in this database was by no means exhaustive, it may indicate that AIL7 elicits the transcriptional regulation of FA biosynthetic and TAG accumulation-related genes by acting upstream of other TFs in the lipid biosynthetic network.

Interestingly, many of the enzyme-encoding fatty acid biosynthetic and TAG accumulation-related genes that were differentially expressed in AIL7-OE lines are also known to be transcriptionally regulated by LAFL TFs and their TF targets (Kumar et al. 2020). These include, for example, *KAS1*, *ACP1*, *FAD3*, *FAE1* and *PDAT2* (Table 1). This insinuates that AIL7 may possibly function within the LAFL transcriptional cascade. While additional research will be required to demonstrate this with certitude, if this were indeed the case, AIL7 would not be the only AIL protein to function in this manner. For example, BBM has been shown to bind and/or transcriptionally activate LEC1, LEC2, ABI3 and AGL15

(directly), as well as FUS3 (indirectly), while PLT2 directly activates LEC1, LEC2 and FUS3 (Horstman et al. 2017). This suggests that these two AIL proteins function directly upstream of LAFL/AGL15 genes.

While no shifts in the expression of most known regulators of seed maturation and fatty acid biosynthesis (e.g., LEC1, LEC2, FUS3, ABI3, ABI4, ABI5, L1L, AGL15, MUM4, GL2, TT2, TT8, ASIL1, TTG1, MYB89, MYB96, MYB118, WRKY6, bZIP67, VAL2, PKL and WRI1) were noted in developing siliques from our AIL7-OE lines (Table S4), significant up-regulation of *VAL1* was observed (Table 1). This gene encodes a B3 domain protein that functions to silence the MADS-box gene *AGL15* (Chen et al. 2018), which is known to control *LEC2* expression through a positive feedback loop (Fatihi et al. 2016). However, the up-regulation of *VAL1* in AIL7-OE lines did not elicit any alteration in the expression of *AGL15* or *LEC2*. Similarly, the up-regulation of *BBM* and *PLT2* in AIL7-OE lines (Table S6) did not provoke any associated increases in *LAFL/AGL15* expression, suggesting that other, as of yet unidentified, TFs within the cascade may be associated with the lipid-related phenotype.

There is also evidence that at least certain AIL proteins may function downstream of LAFL proteins. For example, *BBM* expression is reduced in *lafl* mutant seeds (Horstman et al. 2017), and it has been found previously that an ABI3-like factor from *Phaseolus vulgaris* can bind and activate Arabidopsis *AIL5*, the down-regulation of which led to changes in the expression of several seed maturation-related genes (Sundaram et al. 2013). In addition, *Arabidopsis* FUS3 has been shown to bind *BBM*, *PLT2*, *AIL6* and *AIL7* promoters (Wang and Perry 2013), and up-regulation of *AIL5*, *AIL6* and *AIL7* has been observed in *Arabidopsis* seedlings following the induced over-expression of *FUS3* (Zhang et al. 2016). A search for putative direct gene targets of a small number of available TFs with known functions in TAG accumulation in the Plant Cistrome Database in this study indicated that *AIL7* may also be a direct target of AGL15 and ABI5, the latter of which functions synergistically with ABI4 in the activation of *DGAT1* in Arabidopsis seedlings under stress conditions (Kong et al. 2013). However, further investigation will be required to elucidate the precise positioning and role of AIL7 in the lipid biosynthetic regulatory cascade.

Seeds from AIL7-OE lines also displayed abnormalities in terms of morphology and seedling growth. While AIL7-OE seeds did not exhibit consistent discrepancies in weight or area compared to wild-type seeds (Fig. 3C, D), and both AIL7-OE and wild-type plants possessed seed coats with epidermal cells exhibiting a characteristic reticulated pattern with central columellae, unlike their wild-type counterparts, *AIL7* over-expressing seeds were twisted in shape rather than elliptical, which suggests a defect in morphological differentiation (Fig. 3). A similar twisted seed phenotype has been observed in *Arabidopsis* plants bearing defects in the embryonic cuticle or extra-cuticular sheath at the embryo surface, which leads to the improper separation of the embryo and endosperm during embryo elongation (Kazaz et al. 2020; Moussu et al. 2017). For example, mutations in several genes involved in embryonic cuticle deposition, including the simultaneous disruption/reduction in activity of three Δ9 stearoyl-ACP desaturases (*FATTY ACID BIOSYNTHESIS 2* [*FAB2*], *ACYL-ACYL CARRIER PROTEIN 1* and *5* [*AAD1* and *AAD5*]), as well as *zhoupi* (*zou*), *abnormal leaf shape1* (*ale1*), *twisted seed1* (*tws1*) and *gassho1/2* (*gso1/2*) mutants, have been found to display an embryo/endosperm adhesion phenotype, which presents as twisted seeds (Creff et al. 2019; Fiume et al. 2016; Kazaz et al. 2020; Xing et al. 2013). Similarly, mutations in the *KERBEROS* (*KRS*) gene lead to a very similar phenotype, which has been suggested to occur due to a deficiency in the production of the embryonic sheath rather than the cuticle itself (Moussu et al. 2017). While none of these genes were differentially expressed in developing siliques from AIL7-OE lines (Supplemental Data S1), the substantial alteration in FA composition seen in transgenic seeds compared to wild type (Figs. 2A and S2A) could feasibly have led to cuticle defects in the seed. However, further analyses will be required to establish this definitively.

Morphological defects were also observed in early vegetative and root development in AIL7-OE seedlings compared to wild-type controls, with AIL7-OE seedlings exhibiting reductions in the growth of aboveground vegetative organs, along with shorter primary roots. This decrease in initial seedling growth may be attributable to the decrease in seed oil content seen in these lines, which is akin to deficits in seedling establishment observed in *wri1* mutants that presumably result from a sparsity of seed storage oil (Cernac et al. 2006). However, it is also possible that the defects in seedling growth that occur with *AIL7* over-expression stem from its known functions in meristematic-related processes [(Mudunkothge and Krizek 2012); Table S7] or embryo development [(Horstman et al. 2017); Table S11]. In line with this, *plt1plt2ail6* triple mutants have been reported to exhibit an aberrant organization of the embryonic root pole, resulting in rootless seedlings (Aida et al. 2004). Although the *napin* promoter used to drive the expression of the *AIL7* coding sequence in the transgenic cassette in this study has been shown previously to be active specifically in seeds (Ellerström et al. 1996; Stålberg et al. 1993), we cannot rule out at this point that some level of leaky expression in seedlings may have also contributed to these morphological abnormalities.

In addition to its roles in FA biosynthesis, seed oil accumulation, and seed/seedling growth, our RNA-Seq data suggest that *AIL7* may also have other roles in

processes with potential agronomic importance, such as photosynthesis and stress response (Tables S8 and S9). Interestingly, all DEGs related to photosynthesis were up-regulated in AIL7-OE lines. This is reminiscent of other genes functioning within the LAFL regulatory network, such as *LEC1* and *FUS3*, which have also been found to positively regulate genes involved in photosynthesis either directly or indirectly (Pelletier et al. 2017; Yamamoto et al. 2010). Similarly, various genes with roles in photosynthesis and defense response have also been observed to be up-regulated in *ant ail6* inflorescences (Krizek et al. 2016), which may correlate with the fact that *AIL6* was found to be significantly down-regulated in the developing siliques of AIL7-OE lines (Table S6). However, it remains to be determined whether the up-regulation of such genes translates into increased photosynthetic efficiency/capacity and/or improvements in stress tolerance in developing seeds and/or siliques in the AIL7-OE lines.

Taken together, our data demonstrate that in addition to its known functions in meristem development and maintenance, as well as cell proliferation and shoot phyllotaxy, *AIL7* also plays an important role in seed FA biosynthesis and oil accumulation. While the precise mechanisms through which *AIL7* regulates these processes remain to be unraveled, our findings suggest that like BBM and PLT2, AIL7 may function within the LAFL regulatory cascade. Moreover, our RNA-Seq data suggests additional possible functions for *AIL7* in starch metabolism, embryonic cuticle deposition, photosynthesis and stress response. However, it remains to be determined whether such transcriptional changes translate into alterations in the traits themselves in AIL7-OE lines, and therefore further verification of phenotypic and physiological assessments based on our transcriptomic data will be essential for confirming such roles. These findings not only enhance our understanding of the regulatory network controlling lipid biosynthesis in seeds, but also hint at additional functions of *AIL7*, which could benefit downstream breeding and/or metabolic engineering endeavors.

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Author contribution SDS and GC were responsible for project conception; RJW supervised experiments; SDS and KNJ carried out all molecular, phenotypic and lipid-related studies; CJ carried out bioinformatic analyses; SDS wrote the article with contributions from all authors. SDS and GC agree to serve as the authors responsible for contact and ensure communication.

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Data availability The RNA-Seq data sets are available at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (Accession no. PRJNA725102).

Declarations

Conflict of interest The authors declare no conflicts of interests.

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References

Aida M, Beis D, Heidstra R, Willemsen V, Blilou I, Galinha C, Nussaume L, Noh YS, Amasino R, Scheres B (2004) The *PLETHORA* genes mediate patterning of the Arabidopsis root stem cell niche. Cell 119:109–120

Baud S, Mendoza MS, To A, Harscoet E, Lepiniec L, Dubreucq B (2007) WRINKLED1 specifies the regulatory action of LEAFY COTYLEDON2 towards fatty acid metabolism during seed maturation in Arabidopsis. Plant J 50:825–838

Baud S, Dubreucq B, Miquel M, Rochat C, Lepiniec L (2008) Storage reserve accumulation in Arabidopsis: metabolic and developmental control of seed filling. Arabidopsis Book/am Soc Plant Biol 6:e0113

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc: Ser B (methodol) 57:289–300

Cernac A, Andre C, Hoffmann-Benning S, Benning C (2006) WRI1 is required for seed germination and seedling establishment. Plant Physiol 141:745–757

Chen M, Du X, Zhu Y, Wang Z, Hua S, Li Z, Guo W, Zhang G, Peng J, Jiang L (2012) Seed *Fatty Acid Reducer* acts downstream of gibberellin signalling pathway to lower seed fatty acid storage in Arabidopsis. Plant Cell Environ 35:2155–2169

Chen G, Woodfield HK, Pan X, Harwood JL, Weselake RJ (2015) Acyl-trafficking during plant oil accumulation. Lipids 50:1057–1068

Chen N, Veerappan V, Abdelmageed H, Kang M, Allen RD (2018) HSI2/VAL1 silences AGL15 to regulate the developmental transition from seed maturation to vegetative growth in Arabidopsis. Plant Cell 30:600–619

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Creff A, Brocard L, Joubes J, Taconnat L, Doll NM, Marsollier AC, Pascal S, Galletti R, Boeuf S, Moussu S, Widiez T, Domergue F, Ingram G (2019) A stress-response-related inter-compartmental

signalling pathway regulates embryonic cuticle integrity in Arabidopsis. PLoS Genet 15:e1007847
Cross JM, Clancy M, Shaw JR, Greene TW, Schmidt RR, Okita TW, Hannah LC (2004) Both subunits of ADP-glucose pyrophosphorylase are regulatory. Plant Physiol 135:137–144
Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 139:5–17
Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H, Stymne H (2000) Phospholipid:diacylglycerol acyltransferase: An enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc Natl Acad Sci USA 97:6487–6492
Du Y, Scheres B (2017) PLETHORA transcription factors orchestrate de novo organ patterning during Arabidopsis lateral root outgrowth. Proc Natl Acad Sci USA 114:11709–11714
Eastmond PJ (2006) SUGAR-DEPENDENT1 encodes a patatin domain triacylglycerol lipase that initiates storage oil breakdown in germinating Arabidopsis seeds. Plant Cell 18:665–675
Ellerstrom M, Stalberg K, Ezcurra I, Rask L (1996) Functional dissection of a napin gene promoter: identification of promoter elements required for embryo and endosperm-specific transcription. Plant Mol Biol 32:1019–1027
Eskin NAM, Vaisey-Genser M, Durance-Todd S, Przybylski R (1989) Stability of low linolenic acid canola oil to frying temperatures. J Am Oil Chem Soc 66:1081–1084
Fatihi A, Boulard C, Bouyer D, Baud S, Dubreucq B, Lepiniec L (2016) Deciphering and modifying LAFL transcriptional regulatory network in seed for improving yield and quality of storage compounds. Plant Sci 250:198–204
Fiume E, Guyon V, Remoué C, Magnani E, Miquel M, Grain D, Lepiniec L (2016) TWS1, a novel small protein, regulates various aspects of seed and plant development. Plant Physiol 172:1732–1745
Focks N, Benning C (1998) Wrinkled1: a novel, low-seed-oil mutant of Arabidopsis with a deficiency in the seed-specific regulation of carbohydrate metabolism. Plant Physiol 118:91–101
Gao H, Gao Y, Zhang F, Liu B, Ji C, Xue J, Yuan L, Li R (2021) Functional characterization of an novel acyl-CoA: diacylglycerol acyltransferase 3–3 (CsDGAT3-3) gene from *Camelina sativa*. Plant Sci 303:110752
Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol Biol 42:819–832
Horstman A, Willemsen V, Boutilier K, Heidstra R (2014) AINTEGUMENTA-LIKE proteins: hubs in a plethora of networks. Trends Plant Sci 19:146–157
Horstman A, Li M, Heidmann I, Weemen M, Chen B, Muino JM, Angenent GC, Boutilier K (2017) The BABY BOOM transcription factor activates the LEC1-ABI3-FUS3-LEC2 network to induce somatic embryogenesis. Plant Physiol 175:848–857
Huai D, Zhang Y, Zhang C, Cahoon EB, Zhou Y (2015) Combinatorial effects of fatty acid elongase enzymes on nervonic acid production in *Camelina sativa*. PLoS ONE 10:e0131755
Huang AHC (2018) Plant lipid droplets and their associated proteins: potential for rapid advances. Plant Physiol 176:1894–1918
Jain T (2020) Fatty acid composition of oilseed crops: a review. In: Thakur M, Modi VK (eds) Emerging technologies in food science: focus on the developing world. Springer Singapore, Singapore, pp 147–153
James DW Jr, Lim E, Keller J, Plooy I, Ralston E, Dooner HK (1995) Directed tagging of the Arabidopsis *FATTY ACID ELONGATION1* (*FAE1*) gene with the maize transposon activator. Plant Cell 7:309–319
Jofuku KD, Omidyar PK, Gee Z, Okamuro JK (2005) Control of seed mass and seed yield by the floral homeotic gene *APETALA2*. Proc Natl Acad Sci USA 102:3117–3122
Josefsson LG, Lenman M, Ericson ML, Rask L (1987) Structure of a gene encoding the 1.7 S storage protein, napin, from *Brassica napus*. J Biol Chem 262:12196–12201
Joung JG, Corbett AM, Fellman SM, Tieman DM, Klee HJ, Giovannoni JJ, Fei Z (2009) Plant MetGenMAP: an integrative analysis system for plant systems biology. Plant Physiol 151:1758–1768
Katche E, Quezada-Martinez D, Katche EI, Vasquez-Teuber P, Mason AS (2019) Interspecific hybridization for Brassica crop improvement. Crop Breed Genetics Genomics 1:e190007
Kazaz S, Barthole G, Domergue F, Ettaki H, To A, Vasselon D, De Vos D, Belcram K, Lepiniec L, Baud S (2020) Differential activation of partially redundant Δ9 stearoyl-ACP desaturase genes is critical for omega-9 monounsaturated fatty acid biosynthesis during seed development in Arabidopsis. Plant Cell 32:3613–3637
Kelly AA, Quettier A-L, Shaw E, Eastmond PJ (2011) Seed storage oil mobilization is important but not essential for germination or seedling establishment in Arabidopsis. Plant Physiol 157:866–875
Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14:R36
Kong Y, Chen S, Yang Y, An C (2013) ABA-insensitive (ABI) 4 and ABI5 synergistically regulate DGAT1 expression in Arabidopsis seedlings under stress. FEBS Lett 587:3076–3082
Kong Q, Yuan L, Ma W (2019) WRINKLED1, a "master regulator" in transcriptional control of plant oil biosynthesis. Plants 8:238
Kong Q, Singh SK, Mantyla JJ, Pattanaik S, Guo L, Yuan L, Benning C, Ma W (2020a) Teosinte branched1/cycloidea/proliferating cell factor4 interacts with wrinkled1 to mediate seed oil biosynthesis. Plant Physiol 184:658–665
Kong Q, Yang Y, Low PM, Guo L, Yuan L, Ma W (2020b) The function of the WRI1-TCP4 regulatory module in lipid biosynthesis. Plant Signal Behav 15:1812878
Krizek BA (2015) *AINTEGUMENTA-LIKE* genes have partly overlapping functions with *AINTEGUMENTA* but make distinct contributions to *Arabidopsis thaliana* flower development. J Exp Bot 66:4537–4549
Krizek BA, Bequette CJ, Xu K, Blakley IC, Fu ZQ, Stratmann JW, Loraine AE (2016) RNA-Seq links the transcription factors *AINTEGUMENTA* and *AINTEGUMENTA-LIKE6* to cell wall remodeling and plant defense pathways. Plant Physiol 171:2069–2084
Kumar N, Chaudhary A, Singh D, Teotia S (2020) Transcriptional regulation of seed oil accumulation in *Arabidopsis thaliana*: role of transcription factors and chromatin remodelers. J Plant Biochem Biotechnol 29:754–768
Le BH, Cheng C, Bui AQ, Wagmaister JA, Henry KF, Pelletier J, Kwong L, Belmonte M, Kirkbride R, Horvath S, Drews GN, Fischer RL, Okamuro JK, Harada JJ, Goldberg RB (2010) Global analysis of gene activity during Arabidopsis seed development and identification of seed-specific transcription factors. Proc Natl Acad Sci USA 107:8063–8070
Lemieux B, Miquel M, Somerville C, Browse J (1990) Mutants of Arabidopsis with alterations in seed lipid fatty acid composition. Theor Appl Genet 80:234–240
Lin Y, Ulanov AV, Lozovaya V, Widholm J, Zhang G, Guo J, Goodman HM (2006) Genetic and transgenic perturbations of carbon reserve production in Arabidopsis seeds reveal metabolic interactions of biochemical pathways. Planta 225:153–164
Lu CF, Napier JA, Clemente TE, Cahoon EB (2011) New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. Curr Opin Biotechnol 22:252–259

Mansfield SG, Briarty LG (1992) Cotyledon cell development in *Arabidopsis thaliana* during reserve deposition. Can J Bot 70:151–164
Moussu S, Doll NM, Chamot S, Brocard L, Creff A, Fourquin C, Widiez T, Nimchuk ZL, Ingram G (2017) ZHOUPI and KERBEROS mediate embryo/endosperm separation by promoting the formation of an extracuticular sheath at the embryo surface. Plant Cell 29:1642–1656
Msanne J, Kim H, Cahoon EB (2020) Biotechnology tools and applications for development of oilseed crops with healthy vegetable oils. Biochimie 178:4–14
Mudunkothge JS, Krizek BA (2012) Three Arabidopsis AIL/PLT genes act in combination to regulate shoot apical meristem function. Plant J 71:108–121
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
Nadler SG, Tritschler D, Haffar OK, Blake J, Bruce AG, Cleaveland JS (1997) Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences. J Biol Chem 272:4310–4315
Nole-Wilson S, Tranby TL, Krizek BA (2005) AINTEGUMENTA-like (AIL) genes are expressed in young tissues and may specify meristematic or division-competent states. Plant Mol Biol 57:613–628
Ohto MA, Fischer RL, Goldberg RB, Nakamura K, Harada JJ (2005) Control of seed mass by APETALA2. Proc Natl Acad Sci USA 102:3123–3128
O'Malley RC, Carol S-s, Song L, Lewsey MG, Bartlett A, Nery JR, Galli M, Gallavotti A, Ecker JR (2016) Cistrome and epicistrome features shape the regulatory DNA landscape resource cistrome and epicistrome features shape the regulatory DNA landscape. Cell 165:1280–1292
Pelletier JM, Kwong RW, Park S, Le BH, Baden R, Cagliari A, Hashimoto M, Munoz MD, Fischer RL, Goldberg RB, Harada JJ (2017) LEC1 sequentially regulates the transcription of genes involved in diverse developmental processes during seed development. Proc Natl Acad Sci USA 114:E6710–E6719
Prasad K, Grigg SP, Barkoulas M, Yadav RK, Sanchez-Perez GF, Pinon V, Blilou I, Hofhuis H, Dhonukshe P, Galinha C, Mahonen AP, Muller WH, Raman S, Verkleij AJ, Snel B, Reddy GV, Tsiantis M, Scheres B (2011) Arabidopsis PLETHORA transcription factors control phyllotaxis. Curr Biol 21:1123–1128
Rahman H, Singer SD, Weselake RJ (2013) Development of low-linolenic acid *Brassica oleracea* lines through seed mutagenesis and molecular characterization of mutants. Theor Appl Genet 126:1587–1598
Shockey JM, Fulda MS, Browse JA (2002) Arabidopsis contains nine long-chain acyl-coenzyme a synthetase genes that participate in fatty acid and glycerolipid metabolism. Plant Physiol 129:1710–1722
Siloto RMP, Findlay K, Lopez-Villalobos A, Yeung EC, Nykiforuk CL, Moloney MM (2006) The accumulation of oleosins determines the size of seed oilbodies in Arabidopsis. Plant Cell 18:1961–1974
Singer SD, Weselake RJ (2018) Production of biodiesel from plant oils. In: Chen G, Weselake RJ, Singer SD (eds) Plant bioproducts. Springer New York, New York, pp 41–58
Singer SD, Weselake RJ, Rahman H (2014) Development and characterization of low alpha-linolenic acid *Brassica oleracea* lines bearing a novel mutation in a "class a" *FATTY ACID DESATURASE 3* gene. BMC Genet 15:94
Singer SD, Chen G, Mietkiewska E, Tomasi P, Jayawardhane K, Dyer JM, Weselake RJ (2016) Arabidopsis GPAT9 contributes to synthesis of intracellular glycerolipids but not surface lipids. J Exp Bot 67:4627–4638
Stålberg K, Ellerstrom M, Josefsson LG, Rask L (1993) Deletion analysis of a 2S seed storage protein promoter of *Brassica napus* in transgenic tobacco. Plant Mol Biol 23:671–683
Subedi U, Jayawardhane KN, Pan X, Ozga J, Chen G, Foroud NA, Singer SD (2020) The potential of genome editing for improving seed oil content and fatty acid composition in oilseed crops. Lipids 55:495–512
Sundaram S, Kertbundit S, Shakirov EV, Iyer LM, Juricek M, Hall TC (2013) Gene networks and chromatin and transcriptional regulation of the phaseolin promoter in Arabidopsis. Plant Cell 25:2601–2617
Tian B, Lu T, Xu Y, Wang R, Chen C (2019) Identification of genes associated with ricinoleic acid accumulation in *Hiptage benghalensis* via transcriptome analysis. Biotechnol Biofuels 12:1–16
Wang F, Perry SE (2013) Identification of direct targets of FUSCA3, a key regulator of Arabidopsis seed development. Plant Physiol 161:1251–1264
Wang Z, Yang M, Sun Y, Yang Q, Wei L, Shao Y, Bao G, Li W (2019) Overexpressing *Sesamum indicum* L.'s DGAT1 increases the seed oil content of transgenic soybean. Mol Breed 39:101
Weiss SB, Kennedy EP, Kiyasu JY (1960) The enzymatic synthesis of triglycerides. J Biol Chem 235:40–44
Weselake RJ, Taylor DC, Rahman MH, Shah S, Laroche A, McVetty PBE, Harwood JL (2009) Increasing the flow of carbon into seed oil. Biotechnol Adv 27:866–878
Woodfield HK, Fenyk S, Wallington E, Bates RE, Brown A, Guschina IA, Marillia EF, Taylor DC, Fell D, Harwood JL, Fawcett T (2019) Increase in lysophosphatidate acyltransferase activity in oilseed rape (*Brassica napus*) increases seed triacylglycerol content despite its low intrinsic flux control coefficient. New Phytol 224:700–711
Wu G-Z, Xue H-W (2010) Arabidopsis β-Ketoacyl-[Acyl Carrier Protein] Synthase I is crucial for fatty acid synthesis and plays a role in chloroplast division and embryo development. Plant Cell 22:3726–3744
Xing Q, Creff A, Waters A, Tanaka H, Goodrich J, Ingram GC (2013) ZHOUPI controls embryonic cuticle formation via a signalling pathway involving the subtilisin protease ABNORMAL LEAF-SHAPE1 and the receptor kinases GASSHO1 and GASSHO2. Development 140:770–779
Yamamoto A, Kagaya Y, Usui H, Hobo T, Takeda S, Hattori T (2010) Diverse roles and mechanisms of gene regulation by the Arabidopsis seed maturation master regulator FUS3 revealed by microarray analysis. Plant Cell Physiol 51:2031–2046
Zhang LZ, Tan QM, Lee R, Trethewy A, Lee YH, Tegeder M (2010) Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in Arabidopsis. Plant Cell 22:3603–3620
Zhang M, Cao X, Jia Q, Ohlrogge J (2016) FUSCA3 activates triacylglycerol accumulation in Arabidopsis seedlings and tobacco BY2 cells. Plant J 88:95–107