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Overexpression of patatin-related phospholipase AIIIδ altered plant growth and increased seed oil content in camelina

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Summary

Camelina sativa is a Brassicaceae oilseed species being explored as a biofuel and industrial oil crop. A growing number of studies have indicated that the turnover of phosphatidylcholine plays an important role in the synthesis and modification of triacylglycerols. This study manipulated the expression of a patatin-related phospholipase AIIIδ (*pPLAIIIδ*) in camelina to determine its effect on seed oil content and plant growth. Constitutive overexpression of *pPLAIIIδ* under the control of the constitutive cauliflower mosaic 35S promoter resulted in a significant increase in seed oil content and a decrease in cellulose content. In addition, the content of major membrane phospholipids, phosphatidylcholine and phosphatidylethanolamine, in *35S::pPLAIIIδ* plants was increased. However, these changes in *35S::pPLAIIIδ* camelina were associated with shorter cell length, leaves, stems, and seed pods and a decrease in overall seed production. When *pPLAIIIδ* was expressed under the control of the seed specific, β-conglycinin promoter, the seed oil content was increased without compromising plant growth. The results suggest that *pPLAIIIδ* alters the carbon partitioning by decreasing cellulose content and increasing oil content in camelina.

Introduction

Camelina sativa belongs to the Brassicaceae family and has emerged as a promising oilseed crop in Pacific Northwest of the United States and Canada. Camelina oilseeds can be used for both food and nonfood purposes. Camelina seed oil is rich in omega-3 fatty acids, such as 18:3, which have commercial interests for nutritional values (Horn *et al.*, 2013; Kang *et al.*, 2011; Lu and Kang, 2008; Nguyen *et al.*, 2013). Compared with other Brassicaceae species, camelina seeds have lower levels of glucosinolates which generate toxic intermediates and limit the livestock feed value (Pilgeram *et al.*, 2007). Camelina can grow in low temperature and on marginal lands (Bramm *et al.*, 1990; Zubr, 1997). In addition, camelina requires low levels of nutrients and therefore reducing the input costs of cultivation (Pilgeram *et al.*, 2007). Camelina reaches maturity within approximately 3 months and is amenable to *Agrobacterium*-mediated transformation by floral dipping (Lu and Kang, 2008). Based on its commercial values, geographic suitability and engineering feasibility, camelina is a crop worthy for improving oil content and quality (Horn *et al.*, 2013; Kang *et al.*, 2011; Lu and Kang, 2008; Nguyen *et al.*, 2013).

Triacylglycerols (TAG) in seed oils are assembled in the endoplasmic reticulum (ER) while fatty acids, the building blocks of TAG, are synthesized in the plastid (Bates *et al.*, 2013; Chapman and Ohlrogge, 2012). The newly synthesized fatty acids in plastids have chain lengths of 16 carbons and 18 carbons with no or one double bonds, while the abundant fatty acyl chains in TAG are 18 carbons and 20 carbons with one to three double bonds (Bates *et al.*, 2013; Chapman and Ohlrogge, 2012). Phosphatidylcholine (PC) is an important intermediate involved in fatty acid trafficking and modification, as well as the assembly of TAG (Chapman and Ohlrogge, 2012; Lu *et al.*, 2009, 2011). For example, it has been proposed that lysophosphatidylcholine (LPC):acyl-CoA acyltransferase (LPCAT) produces PC using plastid-derived fatty acids and shuffles the fatty acids from the plastid to the ER (Wang *et al.*, 2012). PC can be directly used for TAG production and is the substrate in the ER for fatty acid desaturation to synthesize polyunsaturated fatty acids. Phospholipase As (PLAs) can hydrolyse PC to release modified fatty acids and generate LPC for reacylation. Thus, the PLA reaction might be involved in lipid production through shuffling fatty acids and remodelling glycerolipids (Chapman and Ohlrogge, 2012). Recent studies indicate that a patatin-related phospholipase, *pPLAIIIδ*, is involved in the PC turnover and seed oil production in model plant Arabidopsis (Li *et al.*, 2013).

In Arabidopsis, there are 10 *pPLAs* that are classified as *pPLAI*, *pPLAIIα*, β, γ, δ, ε and *pPLAIIIα*, β, γ, δ (Scherer *et al.*, 2010). *pPLAI* and *pPLAIIs* have been reported to be involved in pathogen defences (La Camera *et al.*, 2005, 2009; Yang *et al.*, 2007). The expression of *pPLAIIα* is up-regulated by various abiotic stresses (Matos *et al.*, 2008; Rietz *et al.*, 2004), and *pPLAIIα* deficiency results in a compromised growth under drought stress (Yang *et al.*, 2012). Functions of *pPLAIIγ,* δ and ε have been investigated in Arabidopsis root in responses to hormones and phosphorus starvation (Rietz *et al.*, 2010). Distinctive from pPLAI and pPLAIIs, pPLAIIIs contain the noncanonical esterase GXGXG motif (Li *et al.*, 2011). Recent analyses show that pPLAIIIβ and pPLAIIIδ hydrolyse PC to produce LPC and free fatty acids (Li *et al.*, 2011, 2013). Deficiency of *pPLAIIIβ* in Arabidopsis leads to an increased

hypocotyl and primary root length at early seedling stages whereas overexpression of it results in a decrease in plant height (Li *et al.*, 2011). Activation-tagging of *pPLAIIIδ* leads to a stunted plant growth (Huang *et al.*, 2001). These studies indicate that pPLAs play roles in plant growth.

Of the four *pPLAIII*s (α, β, γ, δ), T-DNA insertional knockout of *pPLAIIIδ* results in a decrease in seed oil content (Li *et al.*, 2013). Overexpressing *pPLAIIIδ* in Arabidopsis leads to improvement of the seed oil content, with increases in 20- and 22-carbon fatty acids at the expenses of 18-carbon fatty acids in seeds (Li *et al.*, 2013). The results indicate that *pPLAIIIδ* plays a role in fatty acid remodelling and oil production in Arabidopsis. To explore whether *pPLAIIIδ* could enhance the seed oil production in oil crops, we overexpressed *pPLAIIIδ* in camelina to determine its roles in seed oil production and plant growth.

Results

Overexpression of *pPLAIIIδ* results in a decreased longitude growth in vegetative and reproductive tissues

To investigate the function of *pPLAIIIδ* in camelina, the gene was placed under the control of the cauliflower virus 35S promoter and multiple *35S::pPLAIIIδ* transgenic camelina lines were generated (Figure 1a). These transgenic lines (35S::OEs) consistently displayed a shorter stature than wild type (WT; Figure 1b). Transgene *pPLAIIIδ* was highly expressed in mutants of *35S::pPLAIIIδ* (Figure 1c). The hypocotyl length of *35S::pPLAIIIδ* plants was 35% shorter than WT as shown in 2-week-old plants (Figure 1d). *35S::pPLAIIIδ* and WT plants had similar number of leaves but displayed more compact rosette (Figure 1e). The leaf length was 40% shorter and 10% wider in *35S::pPLAIIIδ* than that of WT (Figure 1f,i). At 6-week stage, *35S::pPLAIIIδ* plants were 50% shorter (Figure 1g,k), but the stem diameter of the transgenic plants was 35% wider than WT (Figure 1h,j). These results indicate that overexpression of *pPLAIIIδ* inhibits the longitudinal growth of vegetative tissues of camelina.

In reproductive tissues, the flower rosettes, which contain many buds in different developing stages, were much more compact in *35S::pPLAIIIδ* plants than in WT (Figure S1a). The buds of *35S::pPLAIIIδ* were 32% shorter and 16% wider than that of WT (Figure S1b–d). The siliques of *35S::pPLAIIIδ* were 26% shorter and 6% wider than those of WT (Figure S1e–g). The pedicels of *35S::pPLAIIIδ* were 47% shorter and 60% wider than those of WT (Figure S1h,i). The shorter pedicels and rounder

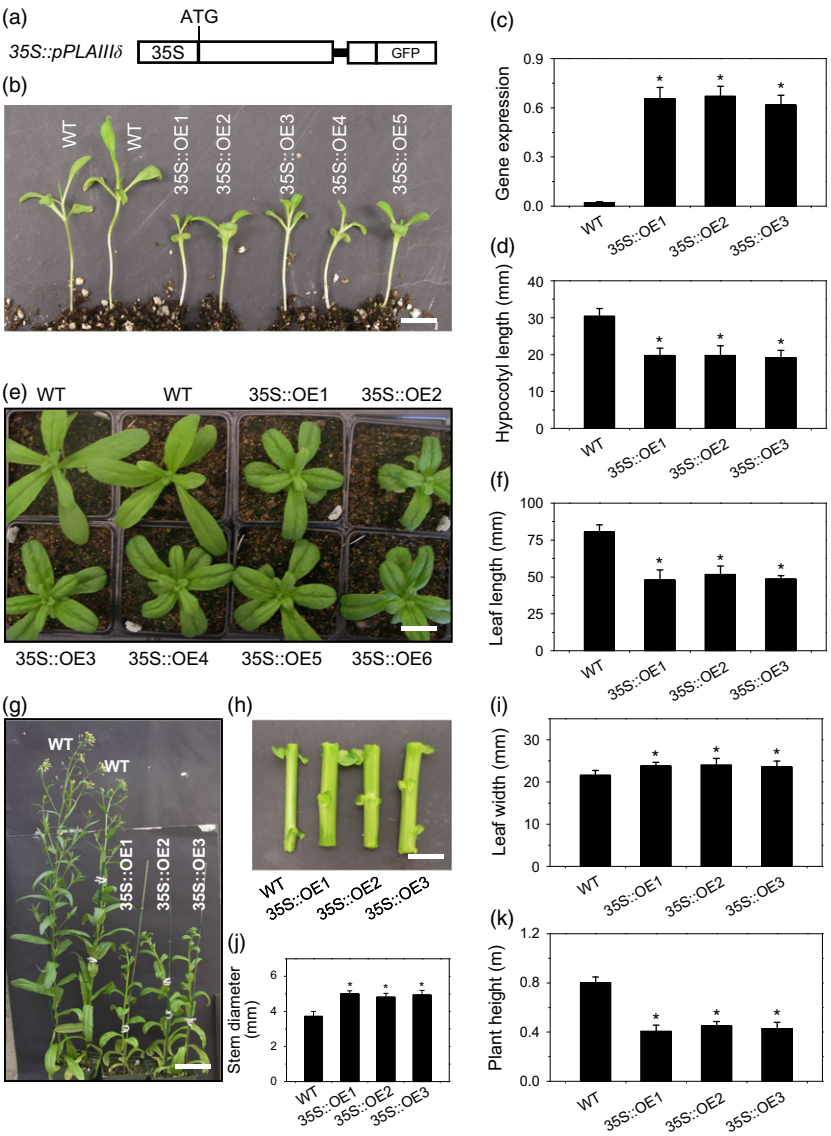


Figure 1 The morphological alterations of the vegetative tissues in camelina mutants overexpressing *pPLAIIIδ*. (a) Construct for overexpressing *pPLAIIIδ* in camelina. The Arabidopsis *pPLAIIIδ* genomic DNA sequence was cloned into a vector with the constitutive expression promoter 35S and in frame with GFP tag at the C-terminus. (b) Morphology of the 2-week-old plants, showing the hypocotyl length. WT: wild-type camelina plants; 35S:OE1,2,3,4,5: independent overexpression lines in T4 generation. Bar = 10 mm. (c) Gene expression of *pPLAIIIδ*. Values are means ± SE (n = 3). (d) Hypocotyl length of the 2-week-old camelina plants. (e) Morphology of the 3-week-old plants, showing the length and width of leaves. Bar = 40 mm. (f) Leaf length of the 3-week-old camelina plants. (g) Morphology of the 6-week-old camelina plants, showing the height of the plants. Bar = 40 mm. (h) Stem width of the 6-week-old camelina plants. Bar = 10 mm. (i) Leaf width of the 3-week-old camelina plants. (j) Stem diameter of the 6-week-old camelina plants. (k) Plant height of the 6-week-old camelina plants. Values are means ± SE (n = 5 for d, f and i-k). *Significant difference at $P < 0.05$ compared with the WT, based on Student's t-test.

siliques were also observed in the 8-week-old inflorescent stalks (Figure S1j). These results indicate that overexpression of *pPLAIIIδ* inhibits the longitudinal growth of reproductive tissues of camelina.

Overexpression of *pPLAIIIδ* represses cell elongation and cellulose accumulation in tissues

To observe the changes in cell morphology, the epidermal cells of the cotyledon and hypocotyls were examined using scanning electron microscopy (Figure 2). The epidermal cells of WT plants were longer while those of *35S::pPLAIIIδ* mutants were shorter and much wider, with the 'jigsaw' morphology not as obvious as in WT (Figure 2a–c). Similarly, the epidermal cells of the *35S::pPLAIIIδ* hypocotyls were 30% shorter than WT (Figure 2d–f,h). The cell density of cotyledons was 35% lower in *35S::pPLAIIIδ* than in WT (Figure 2g). The shorter and wider cells in the *35S::pPLAIIIδ* were consistent with the shorter and wider morphology of leaves and hypocotyls (Figures 1 and 2). These results indicate that overexpression of *pPLAIIIδ* inhibits the anisotropic growth of cells, which results in shorter and wider stems and leaves.

Cell elongation is a process that requires cellulose production and cell wall synthesis (Taylor, 2008). During the plant handling, it was apparent that *35::pPLAIIIδ* tissues were fragile and lacked mechanical strength. When the stems of 5-week-old plants were physically bent, the stems of *35::pPLAIIIδ* were more easily broken than those of WT (Figure 3a). As the mechanical strength comes from the cell wall, we determined whether the mutant plants have altered content of cellulose, a major component of cell wall. The levels of the cellulose decreased 20%, 8% and 14%, in seedlings, leaves and stems of *35S::pPLAIIIδ* plants, respectively, compared with that of WT (Figure 3b–d). These results indicate that overexpression of *pPLAIIIδ* inhibits the

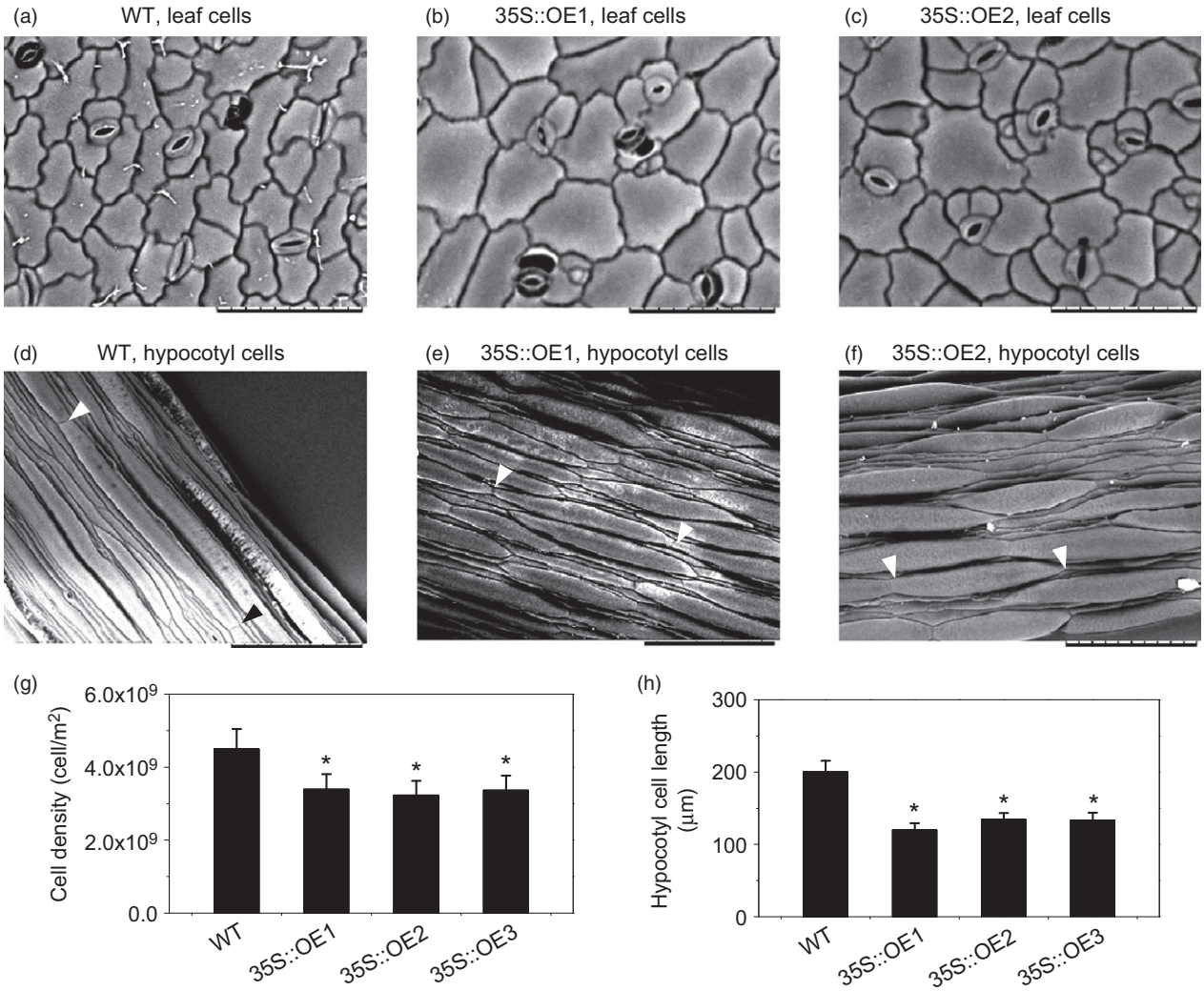


Figure 2 Altered cell length in leaves and hypocotyls of camelina plants overexpressing *pPLAIIIδ*. The epidermal cells of leaves and hypocotyls were examined by scanning electron microscopy. (a–c) Leaf epidermal cells of the 1-week-old camelina plants of WT (a), 35S::OE1 (b) and 35S:OE2 (c), showing the increased width, the reduced cell length and the reduced convolution of epidermal cells in 35S::OE plants compared with that of wild type. (d–f) Hypocotyl cells of the 1-week-old camelina plants of WT (d), 35S::OE1 (e) and 35S::OE2 (f), showing the reduced length and the increased width of epidermal cells in OE plants compared with that of wild type. Arrows mark the beginning and the end of a hypocotyl cell. Bars = 100 μm. (g) Cell density of the leaves. Values are means ± SE (n = 3). (h) Cell length of the hypocotyls. Values are means ± SE (n = 5). *Significant difference at $P < 0.05$ compared with the WT, based on Student's t-test.

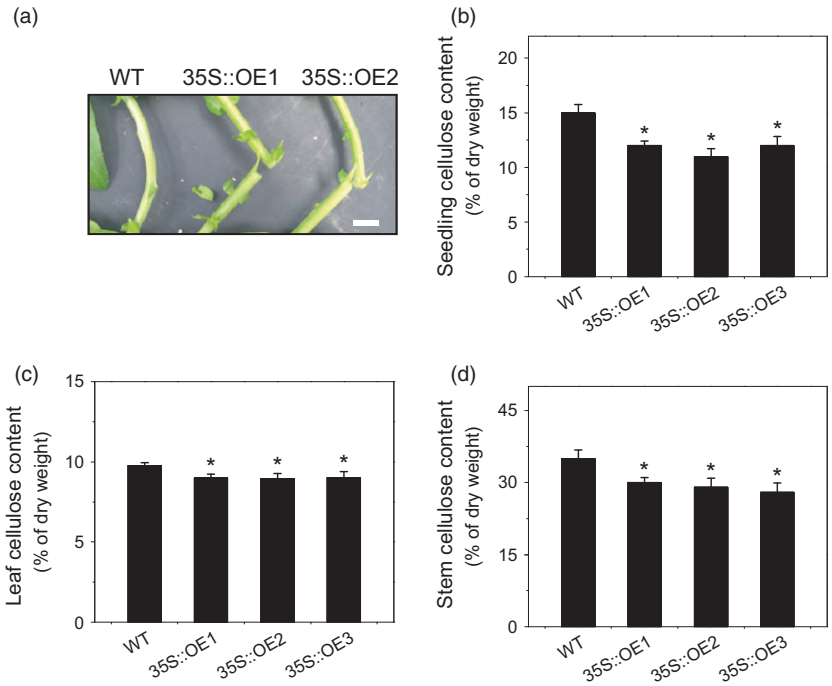


Figure 3 Altered mechanical strength of stems and the reduced cellulose content in tissues of the *pPLAIIIδ* overexpressors of camelina. (a) Physical properties of the primary stem of 5-week-old camelina plants. The *pPLAIIIδ* overexpressors displayed an easily broken stem after bending compared with that of wild type. Bar = 5 mm. (b) Cellulose content of 1-week-old seedlings. (c) Cellulose content of leaves from the 3-week-old plants. (d) Cellulose content of stems from the 5-week-old plants. Values are means ± SE (n = 5). *Significant difference at $P < 0.05$ compared with the WT, based on Student's t-test.

cellulose production, which may impede the mechanical strength of camelina tissues.

Overexpression of *pPLAIIIδ* alters the glycerolipid composition in leaves

The content of glycerolipids in leaves, including phospholipids and galactolipids, were determined by electrospray tandem mass spectrometry (Figures 4 and S2). The levels of PC and phosphatidylethanolamine (PE) were significantly higher while the levels of phosphatidic acid (PA) and phosphatidylglycerol (PG) were significantly lower in *35S::pPLAIIIδ* than in WT (Figure 4). The levels of phosphatidylinositol (PI), phosphatidylserine (PS), monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were similar in WT and *35S::pPLAIIIδ* lines (Figure 4). The total glycerolipids tended to be higher in *35S::pPLAIIIδ* than in WT (Figure 4).

In camelina leaves, PC, PE, PI and PA had similar patterns of molecular species, of which the abundant species were 34C with

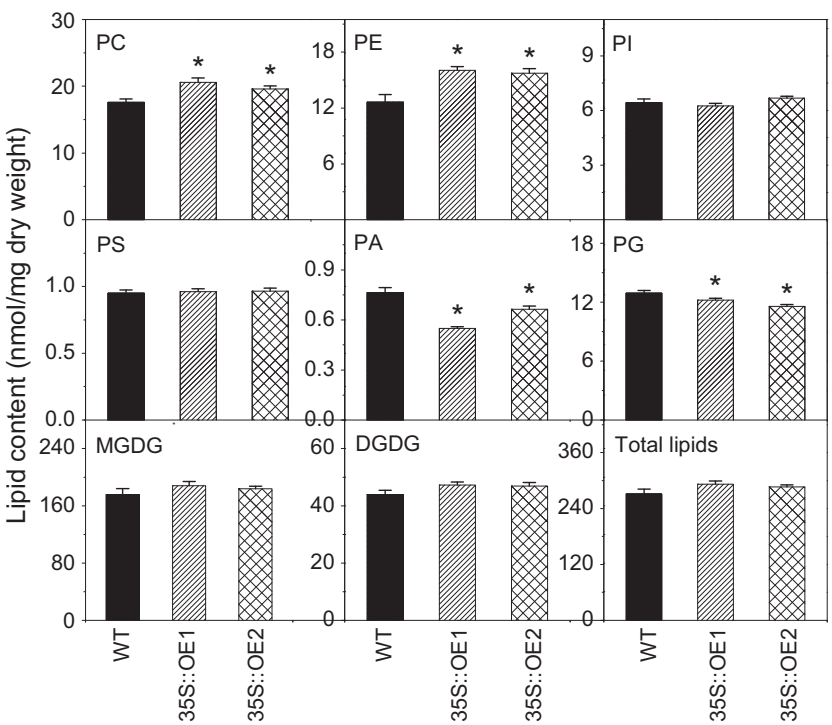


Figure 4 Alterations of glycerolipid levels in leaves of the *pPLAIIIδ* overexpressors. Leaves from the 3-week-old plants were sampled for lipid profiling by ESI-MS/MS. The phospholipid levels, including PC, PE, PI, PS, PA and PG, and the galactolipid levels, including MGDG and DGDG. The total lipid levels referred to the total amount of phospholipids and galactolipids measured. Values are means ± SE (n = 5). *Significant difference at $P < 0.05$ compared with the WT, based on Student's t-test.

1-3 double bonds and 36C with 2-6 double bonds (Figure S2). The MGDG and DGDG had similar molecular patterns, in which the abundant species were 34:6 and 36:6. PS was enriched in very long fatty acyl chains, with 42C as the most abundant species. By comparison, PG was enriched in 34C species with 16C and 18C fatty acyl chains (Figure S2). Most PC and PE species tended to be higher whereas PA and PG species lower in *35S::pPLAIIIδ* than WT plants (Figure S2). The levels of MGDG-36:6 and DGDG-36:6 were higher in *35S::pPLAIIIδ* than in WT (Figure S2).

Overexpression of *pPLAIIIδ* alters the levels of soluble primary metabolites in leaves

As level of glycerolipids in leaves of *35S::pPLAIIIδ* mutants tended to be higher (Figure 4), we wondered whether the carbon source shifting to glycerolipid synthesis will impact levels of other metabolites. The soluble metabolites in the leaves were analysed, and marked changes were observed in the levels of several organic acids, amino acids and soluble sugars in the leaves of *35S::pPLAIIIδ* (Figure 5). The levels of 15 organic acid species were measured, including five species that are related to tricarboxylic acid cycle (TCA cycle), six species that are related to sugar acids, two species that are related to phytohormones and 2 other species (Figure 5a). Most of the organic acids measured displayed a decreased level while some of them had an increased level in mutant leaves (Figure 5a). The levels of three organic acid species in TCA cycle, including malate, fumarate and succinate, were decreased by 53%, 34% and 70%, respectively, in leaves of *35S::pPLAIIIδ* compared with that of WT (Figure 5a). Pyroglutamic acid and propanoic acid are biosynthetically linked to the TCA cycle components of α-ketoglutarate and succinyl-CoA, respectively. The level of pyroglutamic acid increased 117% while the level of propanoic acid increased 22% in *35S::pPLAIIIδ* as compared with that of WT (Figure 5a). The corresponding sugar acids can be derived from sugar species, such as glucose, galactose, ribose, threose, arabinose and mannose. The levels were decreased in *35S::pPLAIIIδ* for glucaric acid (37%), galactaric acid (36%), ribonic acid (27%), threonic acid (59%) and arabinonic acid (43%); the level of mannonic acid was similar in *35S::pPLAIIIδ* and WT (Figure 5a). The levels of two phytohormone-related compounds, benzoic acid and 2-methyl benzoic acid, were twofold higher in *35S::pPLAIIIδ* than in WT (Figure 5a). The levels of 3-butenoic acid increased 49% in *35S::pPLAIIIδ* and WT, and 1,2,3-propanetricarboxylic acid was comparable between two genotypes (Figure 5a).

Five amino acids were determined. Serine, threonine and glycine are biosynthetically linked. The levels of serine and glycine tended to be higher but not significantly in leaves of *35S::pPLAIIIδ* and WT (Figure 5b). The levels of threonine, aspartate and proline decreased 18%, 55% and 33%, respectively, in *35S::pPLAIIIδ* compared with WT (Figure 5b).

Seven of 11 measured monosaccharide species displayed a decreased level in *35S::pPLAIIIδ* compared with WT, including erythrose (40%), lyxose (56%), galactose (36%), mannose (33%), glucopyranose (37%), galactopyranose (29%) and inositol (38%; Figure 5c). The levels of glucose and fructose tended to be 9% and 12% lower in *35S::pPLAIIIδ* compared with WT (Figure 5c). The level of sorbitol had a 20% increase in *35S::pPLAIIIδ* compared with WT (Figure 5c). No change was observed for threitol (Figure 5c). The level of glucopyranosides, a glucose derivative, was 29% lower in *35S::pPLAIIIδ* than in WT (Figure 5c). The levels of disaccharide species, melibiose and maltose, were decreased 45% and 41%, respectively, in *35S::pPLAIIIδ* as compared with WT (Figure 5c). These results indicate that the overexpression of *pPLAIIIδ* has profound impact on carbon metabolism.

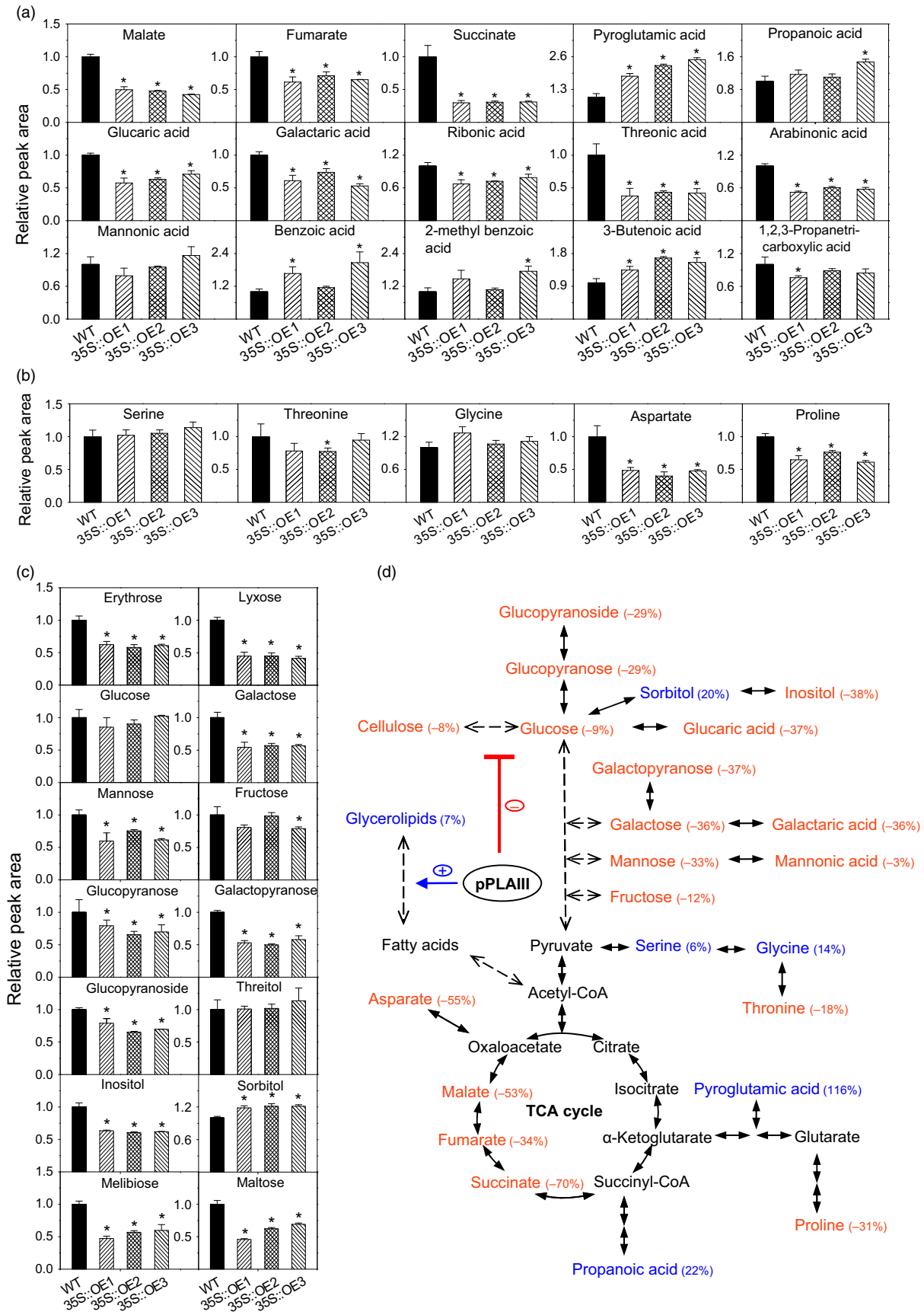
Constitutive overexpression of *pPLAIIIδ* increases seed oil content but decreases seed production

We determined the impact of constitutive overexpression of *pPLAIIIδ* on seed morphology and seed oil content (Figure 6). *35S::pPLAIIIδ* seeds displayed altered seed morphology, being 12% shorter and 26% wider than WT seeds (Figure 6a). As a result, the ratio of width to length was increased 44% in OE seeds compared with WT (Figure 6b). However, the florescent stem of *35S::pPLAIIIδ* plants were approximately 50% shorter with approximately 10% fewer seed pods and the number of seeds per pod decreased approximated 10%. These changes resulted in fewer seeds, with the seed yield per plant of *35S::pPLAIIIδ* being 60% lower than that of WT plants (Figure 6c). *35S::pPLAIIIδ* seeds had a decreased level of 18:1 and an increased level of 20:1 (Figure 6d). The seed oil content was 6.5% higher in *35S::pPLAIIIδ* than in WT seeds (Figure 6e). *35S::pPLAIIIδ* seeds were larger in size and 7% heavier than WT seeds (Figure 6f). Thus, a per seed basis, one *35S::pPLAIIIδ* seed contained 14% more oil than one WT seed. The content of cellulose was 17% lower in *35S::pPLAIIIδ* than in WT seeds (Figure 6g).

Seed-specific overexpression of *pPLAIIIδ* increases seed oil content without compromising plant growth

To overcome the adverse effect on plant growth and seed production, we utilized the seed-specific promoter of β-conglycinin to drive the expression of *pPLAIIIδ* in camelina plants (Figure 7a). We compared the growth of mutants overexpressing *pPLAIIIδ* by 35S constitutive promoter and seed-specific β-conglycinin promoter, designated as 35S::OE and CON::OE, respectively (Figure 7). Analysis of three independent lines in T4 generation showed that *CON::pPLAIIIδ* overexpressors and WT plants had similar morphology at different development stages, such as at 1-, 4 and 5 weeks of growth (Figure 7b–d). No differences were observed in the shape of leaves between *CON::pPLAIIIδ* and WT (Figure 7e). These results suggest that overexpressing *pPLAIIIδ* by seed-specific promoter has no observable impact on plant vegetative growth.

Figure 5 Alterations of metabolite levels in leaves of the *pPLAIIIδ* overexpressors with 35S promoter. Leaves from the 3-week-old plants were sampled for metabolite measurement by GC-MS. (a) The levels of organic acids. (b) The levels of amino acids. (c) The levels of sugar-related compounds. (d) Schematic representation of the primary metabolism pathway in plants and potential function of *pPLAIIIδ*. The dashed line marks directly metabolic linkage. Compounds coloured blue and red indicate a tendency of decreased and increased level, respectively, in *35S::pPLAIIIδ* overexpressors compared with that of WT. TCA cycle, tricarboxylic acid cycle. Values are means ± SE ($n = 5$). *Significant difference at $P < 0.05$ compared with the WT, based on Student's *t*-test.



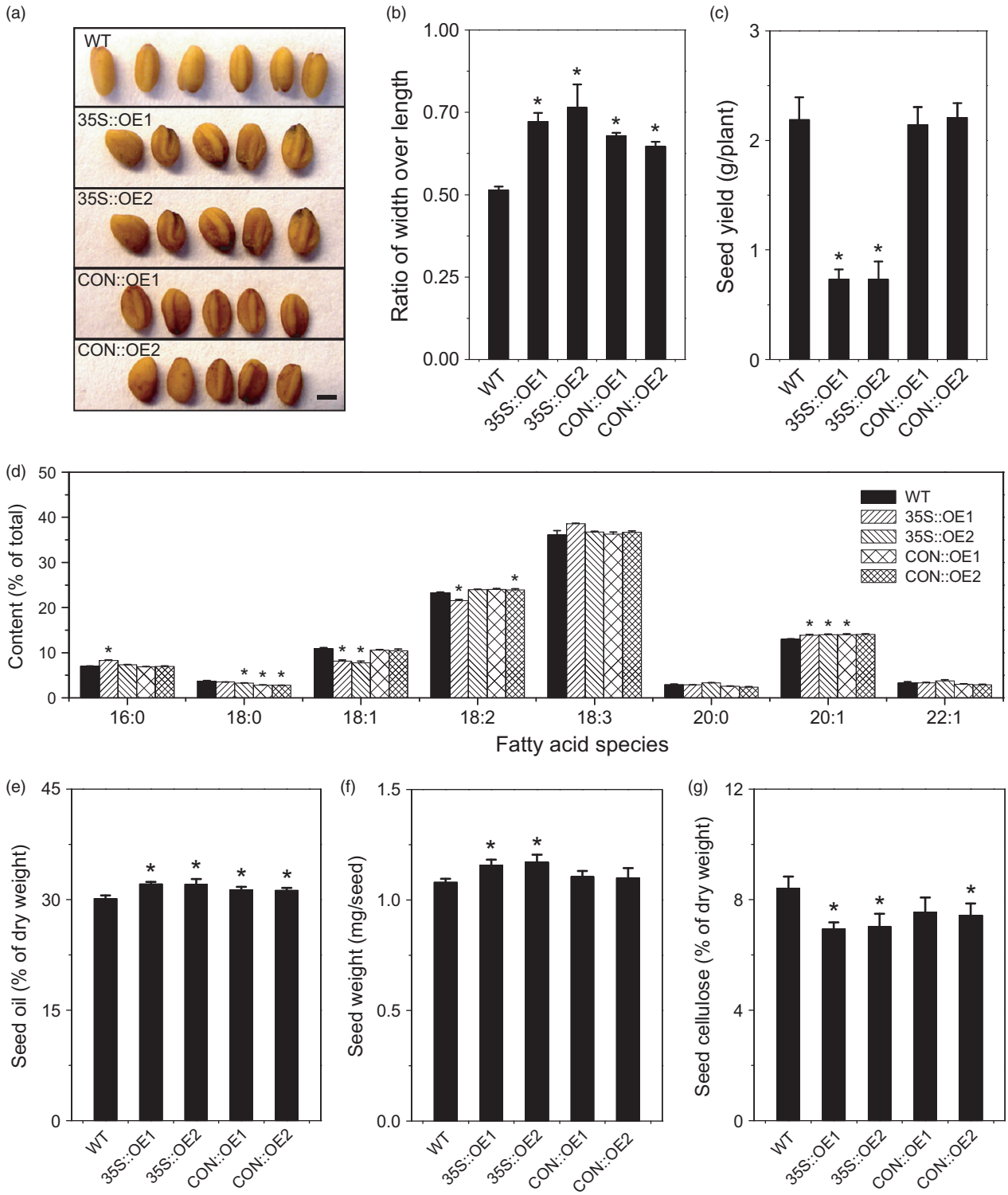


Figure 6 Characterization of camelina mutant seeds overexpressing *pPLAIIIδ*. (a) Morphology of mature camelina seeds of wild-type and *pPLAIIIδ* overexpressors, showing OE seeds were rounder than WT ones. Bar = 1 mm. WT, wild type; 35S::OE, constitutive overexpressors. CON::OE, seed-specific overexpressors. (b) Ratio of seed width over seed length, an indicator of seed shape. (c) Seed yield of individual plant. (d) Fatty acid compositions. (e) Seed oil content. (f) Weight of individual seed. Five replicates of seed samples from each genotype were weighed and the number of the seeds from each replicate was counted. (g) Seed cellulose content. Values were means ± SE (n = 5 for b-e and g; n = 10 for f). *Significant difference at $P < 0.05$ compared with the WT, based on Student's t-test.

The production of the recombinant protein pPLAIIIδ-GFP was detected in the developing seeds of *CON::pPLAIIIδ* (Figure 7f). The green fluorescence protein (GFP) signal of pPLAIIIδ-GFP was observable at 17 day-after-pollination (DAP), reached the maximum at 21 DAP and maintained the presence till 25 DAP which corresponds to seed maturation (Figure S3). *CON::pPLAIIIδ* seeds

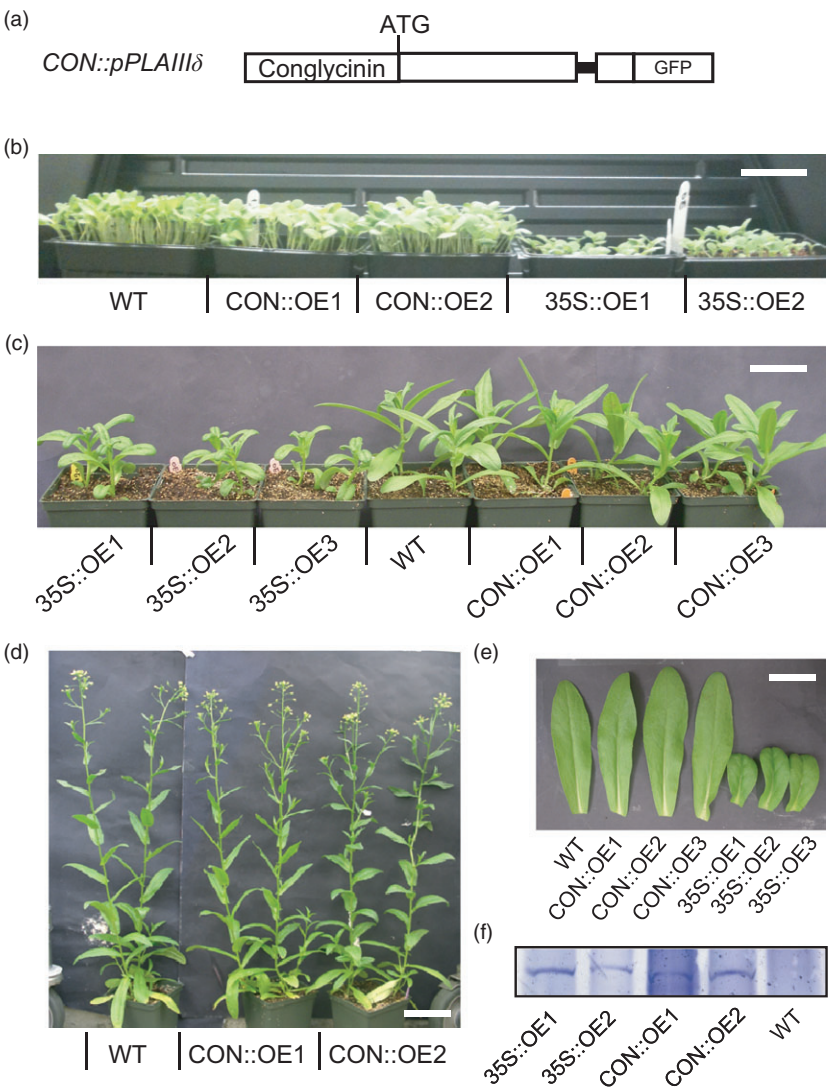


Figure 7 Generation of the seed-specific overexpressors of *pPLAIIIδ* in camelina plants. (a) Construct for seed-specific overexpression of *pPLAIIIδ* in camelina. The Arabidopsis *pPLAIIIδ* genomic DNA sequence was cloned into a vector with the seed-specific expression promoter of conglycinin protein and in frame with GFP tag in the C-terminus. (b) Plant height of the 1-week-old seedlings, showing the similar height of seed-specific overexpressors (CON::OE) and reduced height of constitutive overexpressors (35S::OE) compared with that of wild type. Bar = 50 mm. (c) Plant height of the 24-day-old plants, showing the similar height of CON::OE mutant plants and reduced height of 35S::OE mutant plants compared with the wild-type ones. Bar = 50 mm. (d) Plant height of 5-week-old plants, showing the similar height of WT and CON::OE mutants. Bar = 100 mm. (e) The similar leaf morphology of CON::OE mutants with the wild-type ones. Bar = 20 mm. (f) Western blotting to detect the recombinant protein of pPLAIIIδ-GFP in the developing seeds of the mutant plants.

were 5% shorter and 25% wider than WT ones (Figure 6a). The *CON::pPLAIIIδ* seeds were rounder; the ratio of width over length was 30% greater than WT seeds (Figure 6b). Individual seed weight and seed yield per plant were comparable between *CON::pPLAIIIδ* and WT plants (Figure 6c,f). Seeds from two of the *CON::pPLAIIIδ* lines exhibited a lower level of 18:0 and a slightly lower level of 18:1 (Figure 6d). *CON::pPLAIIIδ* seeds tended to have a higher level of 20:1 (Figure 6d). The *CON::pPLAIIIδ* lines displayed a significant 4% increase in seed oil content compared with WT seeds (Figure 6e).

We determined the harvest index for mutants of *CON::pPLAIIIδ* (Figure 8). For a direct comparison, WT plants and *CON::pPLAIIIδ* mutants were grown in containers with a dimension of length 32 cm, width 32 cm and height 32 cm (Figure S4). The plant dry mass and seeds were harvested, and the seed parameters and harvest index were measured. The seed oil content was approximately 9% higher in *CON::pPLAIIIδ* than in WT (32.7% vs 30.0%; Figure 8a). No significant difference was found in plant height, total dry mass per plant, seed yield per plant and seed starch content between WT and *CON::pPLAIIIδ* overexpressors (Figure S4d–g). The oil yield per plant was approximately 15% higher in *CON::pPLAIIIδ* lines than in WT (Figure 8b; Table S2). The harvest index was significantly higher (14%) in *CON::pPLAIIIδ* lines than in WT (Figure 8c; Table S2). These results indicate the seed-specific overexpression of *pPLAIIIδ* enhances an overall seed oil yield in camelina plants.

The seed yield per plant, seed oil content and oil yield per plant were compared between plants growing in pots and in growth containers (Table S1). Plants growing in pots set more seeds per plant than those in growth containers (Table S1), and this difference may be contributed by the less plant density and thus larger growth area in pots than in growth containers (Table S1). The level of seed yield per area was higher for plants growing in containers than those in pots (Table S1). Under both growth conditions, the seed oil content and oil yield per plant were higher in *CON::pPLAIIIδ* lines than in WT (Table S1). These results indicate enhancement of seed oil content in transgenic plants is due to the overexpression of *pPLAIIIδ*.

Discussion

The present study shows that the overexpression of *pPLAIIIδ* in camelina enhances seed oil content with an increased proportion of long chain fatty acids. These effects of *pPLAIIIδ* in camelina are consistent with recent findings in Arabidopsis (Li *et al.*, 2013), indicating the potential to use *pPLAIIIδ* for improving oil produc-

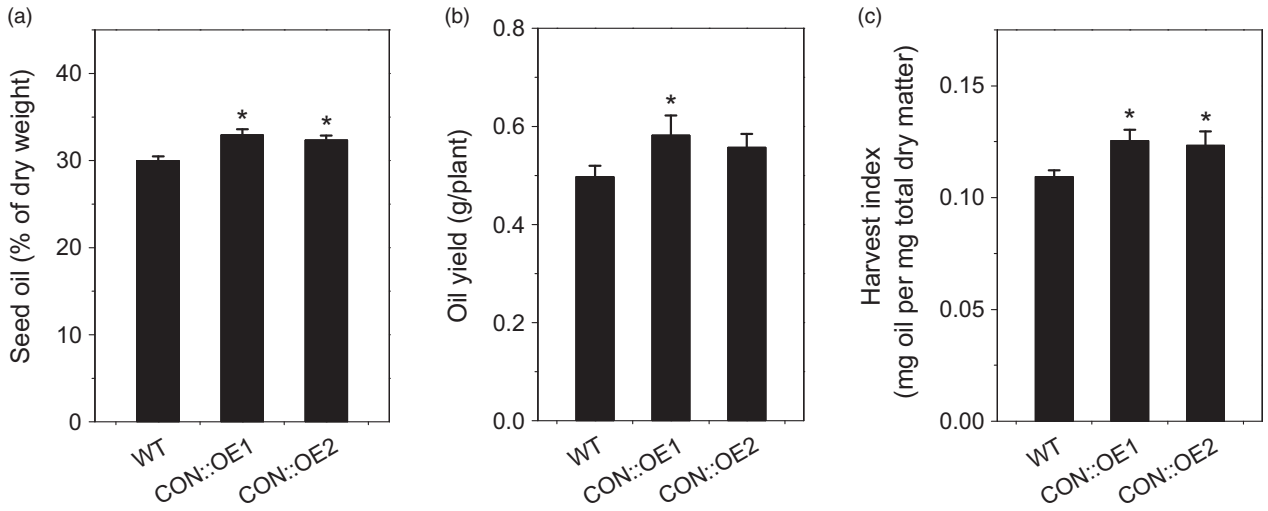


Figure 8 Oil content and harvest index of camelina seed with seed-specific overexpression of *pPLAIIIδ*. (a) seed oil content. (b) Oil yield per plant. (c) Harvest index. WT, wild-type; CON::OE, seed-specific overexpressors. Plants were growing in containers with dimension of length 32 cm, width 32 cm and height 32 cm. Seven WT plants and 7 CON::OE plants grew in one container. The seeds and the total dry plant mass of the 7 individual plants were put together as one sample. Total plant dry mass was from the above-ground parts of the plants, including seeds, dried leaves and dried stems. Values were means ± SE (n = 10 for WT and 5 for CON::OE). *Significant difference at $P < 0.05$ compared with the WT, based on Student's t-test.

tion. While it enhances lipid production, the 35S promoter-driven, constitutive expression of *pPLAIIIδ* is detrimental to biomass and seed production. Seed-specific overexpression of *pPLAIIIδ* increases oil content without compromising overall plant growth and seed production in camelina.

One intriguing question arising from these results is how the glycerolipid-hydrolysing pPLAIIIδ increases lipid accumulation. One hypothesis is that pPLAIIIδ-mediated PC turnover facilitates the movement of fatty acids from the plastid to the ER. Fatty acids in higher plants are synthesized primarily in the plastids and exported to ER for glycerolipid synthesis, including PC, PE and TAG (Bates *et al.*, 2013). pPLAIIIδ hydrolyses PC to produce free fatty acids and LPC (Li *et al.*, 2013). LPC can be reacylated to PC by LPCAT using fatty acyl-CoA derived from the plastids. The LPCAT reaction has been implicated in shuffling fatty acids from the plastid to the ER (Bates *et al.*, 2012; Tjellström *et al.*, 2012). The release of acyl chains from PC to ER is predicted to be catalysed by phospholipase A activities (Lands, 1960; Chapman and Ohlrogge, 2012). pPLAIIIδ may hydrolyse acyl chains from PC and generate LPC as substrate for LPCAT. Therefore, overexpression of *pPLAIIIδ* could enhance the acyl flux into ER and increase the overall levels of glycerolipids, such as PC and PE. Newly formed PC is enriched in fatty acids with chain length of 16 carbons and 18 carbons with no or one double bond (16:0, 18:0, 18:1). These plastid-originated fatty acids in PC are further desaturated to form 18:2 and 18:3. In addition, fatty acids on PC can be released to form acyl-CoA for elongation to form 20 carbon fatty acids. The fatty acid trafficking and modifications, including desaturation and elongation, are very active during oilseed development (Bates *et al.*, 2013). *pPLAIIIδ*-overexpressing seeds tended to have a higher level of 20:1 and polyunsaturated 18 carbon fatty acids at the expense of 16:0, 18:0 and 18:1, which are direct products of fatty acid synthesis in the plastid. These findings suggest *pPLAIIIδ* may play roles in trafficking fatty acids from plastids to ER, as well as in modifying fatty acids to produce the ones with over 20 carbons.

Many compounds of the amino acids, organic acids and sugar species are linked metabolically (Figure 5d). More carbon shifting to glycerolipid synthesis may impact the levels of metabolites in tricarboxylic acid (TCA) cycle. Our results showed that the levels of organic acids in TCA cycle decreased around 50% in *35S::pPLAIIIδ* leaves, such as malate, fumarate and succinate (Figure 5a). The increased demand for glycerolipid synthesis in *35S::pPLAIIIδ* mutant resulted in less carbon entering TCA cycle (Figure 5d). The metabolic relationship of glycerolipid synthesis and TCA cycle is also observed in other mutants. In both *shrunken seed 1* mutant (*sse1*) and mutant overexpressing biotin carboxyl carrier protein 2, the decreased demand for glycerolipid synthesis is accompanied by more carbons entering TCA cycle (Chen *et al.*, 2009; Lin *et al.*, 2006). The decreased levels of metabolites in TCA cycles may impact the levels of amino acids that are metabolically linked to the TCA cycles, such as asparate and proline. Our results showed that the levels of asparate and proline decreased by around 40% (Figure 5a). Cellulose is derived from glucose units, which are linked through β(1→4)-glycosidic bonds. The cellulose level was lower in leaves of *35S::pPLAIIIδ* (Figure 3c), suggesting a less utilization of glucose for cellulose synthesis and more carbon source could be allocated to glycerolipid synthesis (Figure 5d). The overall lower levels of many types of soluble sugars and their derived sugar acids all support more carbons were directed toward lipid production resulting from the increased *pPLAIIIδ* expression (Figure 5c).

An interesting question arising from the overt impact of *pPLAIIIδ* overexpression on plant growth is the role of the *pPLAIII* subfamily in overall plant growth regulation. The impairment in cell elongation and anisotropic growth has been proposed to result from decreased cellulose production (Burk *et al.*, 2001; Fagard *et al.*, 2000). Longitudinal cell growth requires cellulose deposition, and cellulose deficient mutants, such as the *fragile arabidopsis2 (fra2)* and *Cellulose synthase 6*-deficient *prc1-1,* displayed similar loss of anisotropic cell growth (Burk *et al.*, 2001; Fagard *et al.*, 2000). The changed membrane compositions might

impede the function of membrane associated proteins, such as FRA2. FRA2 is a microtubule-severing protein with ATPase activity and is involved in cellulose deposition in Arabidopsis stalks; loss of function mutant, *fra2*, results in decreased levels of cellulose content and mechanical strength (Burk *et al.*, 2001). Fatty acids can inhibit ATPase activities (Lamers and Hülsmann, 1977; Swann, 1984; Swarts *et al.*, 1991). pPLAIIIδ is localized on the plasma and intracellular membranes, and *pPLAIIIδ*-overexpressing Arabidopsis has elevated levels of free fatty acids and lysophospholipids (Li *et al.*, 2013). It might be possible that pPLAIIIδ-derived products, such as fatty acids and LPC, impair the ATPase activity of FRA2. FRA2 is a katanin-like protein (Burk *et al.*, 2007). Suppression of *katanin* expression results in a lower cellulose content and a higher lipid content (Burk *et al.*, 2007; Qu *et al.*, 2012), a consequence similar to overexpression of *pPLAIIIδ*. pPLAIIIδ-derived membrane disturbance may also impact the trafficking and function of the cellulose synthase complex on cellulose deposition. Future investigations on how a higher level of pPLAIIIδ decreases cellulose accumulation may reveal an important regulatory mechanism that coordinates lipid and cellulose metabolism.

Seed-specific overexpressors were tested for growth and seed oil production in pots and growth containers (Figures 6 and 8). In both growth conditions, levels of the seed oil content per weight and oil yield per unit of growth area were higher in seed-specific overexpressors than in WT (Table S1), suggesting a positive impact of *pPLAIIIδ* on seed oil accumulation. The difference in the magnitude of oil increases in pots and growth containers may result from the different growth conditions. In particular, the growth conditions in individual pot tended to be more variable such as soil water content and potentially fertilizer levels because each plant was in a separate pot. By comparison in growth containers, WT and seed-specific overexpressors were growing in a same container so the soil moisture and nutrient levels were the same. In addition, seeds from 7 plants growing in a same growth container were put together and treated as one sample, while seeds from one plant growing in an individual pot was treated as one sample. Therefore, the data for plants growing in growth containers had a smaller variation and were more directly comparable among genotypes than those growing in separated pots.

In summary, our study shows that the ubiquitous overexpression of *pPLAIIIδ* results in a significant increase in seed oil content, but decrease in cellulose accumulation, longitude growth and seed production in camelina plants. Seed-specific overexpression of *pPLAIIIδ* enhances seed oil content without the detrimental effects on the plant growth and seed yield. Targeted manipulation of *pPLAIIIδ* has the potential to improve seed oil production in oilseed crops.

Experimental procedures

Generation of *35S::pPLAIIIδ* and *CON::pPLAIIIδ* overexpression plants

To overexpress *pPLAIIIδ*, the genomic sequence of *pPLAIIIδ* (At3g63200) was obtained by PCR using Col-0 Arabidopsis genomic DNA as a template and by forward primer 5′-ATTTA ATTAAATGGAGATGGATCTCAGCAAGGTT-3′ and reverse primer 5′-ATGGCGCGCCAACGGCCGTCAGCGAGAGGGTTAA-3′. The genomic sequence of *pPLAIIIδ* was cloned into the pMDC83 vector before the GFP-His coding sequence and between the PacI and AscI sites, with the expression of *pPLAIIIδ* under the control of the 35S cauliflower mosaic virus promoter (pMDC83-*35S::pPLAIIIδ*). The β-conglycinin promoter sequence was amplified by forward primer 5′-ATGTTTAAACAAGCTTTTGATCCATGCCCTT-CAT-3′ and reverse primer 5′-ATTTAATTAAGCGGCCGCAGTATA TCTTAAATTCT-3′ and then cloned into pMDC83-*35S::pPLAIIIδ* between the sites of PmeI and PacI. The 35S promoter was replaced by β-conglycinin promoter and a new construct, pMDC83-*CON::pPLAIIIδ* was obtained.

The sequences of the constructs were verified before they were introduced into the *Agrobacterium tumefaciens* strain C58C1. *Camelina sativa* (cv. Suneson) plants were transformed by the pMDC83-*35S::pPLAIIIδ*- or pMDC83-*CON::pPLAIIIδ*-containing *Agrobacterium tumefaciens* using a floral dip technique (Lu and Kang, 2008). Transgenic plants were screened by hygromycin resistance selection and confirmed by PCR using one primer specific to the *pPLAIIIδ* gene and the other specific to the promoter. Homozygous transgenic lines were obtained, and three independent lines for each were further characterized at T4 generation.

Real time PCR was applied to determine the expression of *pPLAIIIδ* in camelina plants. The primers for detecting *pPLAIIIδ* were 5′-CAACGTCTTGTTGCGTCAGGAAAGTC-3′ and 5′-ATTA-ACTCGAAGATGCTGGCTGGG-3′. *CsTubulin* was used as control gene, and the primers were 5′-CACCTCAAGAGGGTCTCAGC-3′ and 5′-ACGTTCAGCATCTGCTCGTC-3′ (Jang *et al.*, 2014; Kwak *et al.*, 2013). The detailed PCR conditions were described previously (Li *et al.*, 2013).

Analysis of fatty acid composition, oil content and harvest index

Ten camelina seeds were placed in glass tubes with Teflon-lined screw caps, and 1.5 mL 5% (v/v) H_2SO_4 in methanol with 0.2% butylated hydroxytoluene was added. The seeds were crushed by a glass pestle, and samples were incubated for 1 h at 90 °C for oil extraction and transmethylation. Fatty acid methyl esters (FAMEs) were extracted with hexane, and quantified using gas chromatography on a SUPELCOWAX-10 (0.25 mm × 30 m) column with helium as a carrier gas at 20 mL/min and detection by flame ionization. The oven temperature was maintained at 170 °C for 1 min and then ramped to 210 °C at 3 °C per min. FAMEs from TAG were identified by comparing their retention times with FAMEs in a standard mixture. Heptadecanoic acid (17:0) was used as the internal standard to quantify the amounts of individual fatty acids. In calculation of harvest index, the dry matter of the above-ground parts of 7 plants from one container was put together and treated as one samples. The wild-type plants had 10 samples and each line of *CON::pPLAIIIδ* had five samples. The oil yield was calculated by multiplying oil content and seed yield. The harvest index was expressed as mg oil per mg of total plant dry matter. The principle for calculating harvest index was followed as reported previously (Nanja Reddy *et al.*, 2003).

Plant growth, lipid profiling and metabolite measurements

Camelina plants were grown in an air-conditioned, climate-controlled (Argus Control Systems) greenhouse set to the following conditions: heating set point of 20 °C; cooling set point of 21 °C; 50% minimum humidity; 16-h light/8-h dark cycle; supplemental light threshold of 566 μmol/m^2/s (supplemental lights turn off when outside solar radiation is 566 μmol/m^2/s or above); 100% shading with shade cloth when solar radiation is 1415 μmol/m^2/s or above; and 50% shading at

1132 μmol/m^2/s or above. Plants were watered regularly and fertilized with Jack's® 15-16-17 Peat-lite water-soluble fertilizer once a week. Each plant was growing in a separate pot (8 × 8 × 8 cm, width × length × height) and 9 pots were put in a tray (26 × 52 cm, width × length). Therefore, each individual plant had a growth area of 150 cm^2 when growing in pots. In the experiments of measuring harvest index, 14 plants were grown in a growth container (32 × 32 × 32 cm, width × length × height) and each individual plant had a growth area of 73 cm^2.

The leaves were harvested from 3-week-old plants for measurement of metabolites. The levels of PA, PC, PE, PG, PI, PS, MGDG and DGDG were determined as previously described (Welti *et al.*, 2002) with modifications described by Xiao *et al.* (2010). Lipid extracts were introduced by continuous infusion into the electrospray source on a triple quadrupole mass spectrometer (API4000; Applied Biosystems, Foster City, CA).

The levels of metabolites of amino acids, organic acids and sugar-related compounds were determined by gas chromatography-mass spectrometry (GC-MS) using the protocol defined by Broeckling *et al.* (2005). The sample was analysed by an Agilent 7820A GC coupled to a 5975 MSD scanning from *m/z* 50-650. A 60 m DB5-MS column (0.25 mm ID, 0.25 *u*m film thickness. J&W Scientific, Santa Clara, CA, USA) was utilized to separate metabolites at a constant flow of 1.0 ml/min and by a temperature programming of a 80 °C for 2 min, ramped at 5 °C/min to 315 °C and held for 12 min.

Microscopy imaging

Images of developing seeds from camelina siliques were captured using a Nikon Eclipse 800 widefield microscope and an ×60 differential interference contrast, 1.2-numerical aperture objective, with mercury lamp excitation, a 492/18 BP excitation filter and a 535/40 B emission filter. For scanning electron microscopy, fresh samples were fixed to specimen stubs using OCT, frozen in liquid nitrogen for 1 min and then immediately transferred to the scanning electron microscope for observation in the frozen state using the backscatter detector of a Hitachi TM 1000 tabletop scanning electron microscope (Hitachi High-Technologies Canada, Inc. Toronto, Ontario, Canada).

SDS-PAGE and immunoblotting

Leaf samples, each weighing approximately 1 g, were harvested and ground in 3 mL buffer of 30 mM HEPES, pH 7.5, 400 mM NaCl, 1.0 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol. The homogenates were centrifuged at 12 000 *g* for 15 min at 4 °C. Supernatants were assayed for protein content, and equal amounts of proteins were separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride filter. The filter was incubated with GFP antibodies overnight. The membrane was visualized with alkaline phosphatase conjugated to a secondary anti-mouse antibody after blotting.

Cellulose and starch measurement

Plant matter was cut into pieces, immersed in liquid nitrogen and ground into powder. The materials were extracted twice with 70% ethanol at 70 °C for 1 h. Cell wall materials were dried under vacuum for cellulose content measurement (Updegraff, 1969). Whatman 3MM filter paper was used to establish a standard curve for quantification of cellulose. Anthrone reagent was used to determine the cellulose content. The protocol for measuring starch content was followed as reported previously (Bahaji *et al.*, 2011; Chow and Landhäusser, 2004; Eimert *et al.*, 1995).

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Authors' contributions

ML designed the study, performed the experiments, analysed the results and wrote the manuscript. WF performed the metabolic analysis. AT, MT and AS participated the genetic cloning, camelina transformation, genetic screening and mutant confirmation. WX designed the study, analysed the results and revised the manuscript. All authors participated in correction of the manuscript and approved the final version.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 The morphological alteration of the reproductive tissues in camelina mutants overexpressing *pPLAIIIδ*.
Figure S2 Profiling of individual species of phospholipids and galactolipids in leaves of wild type and the *pPLAIIIδ* overexpressors.
Figure S3 Detection of pPLAIIIδ protein expression in developing seeds of seed-specific overexpressors.
Figure S4 Camelina plants growing in containers with dimension of length 32 cm, width 32 cm, and height 32 cm.
Table S1 Seed yield, oil content, and oil yield of plants growing in pots and growth containers.
Table S2 Oil content, oil yield and harvest index of plants growing in growth containers.