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SPECIAL ISSUE ARTICLE

Genetically engineering *Crambe abyssinica*—A potentially high-value oil crop for salt land improvement

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Abstract

Crambe abyssinica (crambe) is a new industrial oil crop that can grow on saline soil and tolerates salty water irrigation. Genetically engineered crambe in which the seed-oil composition is manipulated for more erucic acid and less polyunsaturated fatty acid (PUFA) would be highly beneficial to industry. In this research, lysophosphatidic acid acyltransferase 2 RNA interference (CaLPAT2-RNAi) was introduced into the crambe genome to manipulate its oil composition. The result showed in comparison with wild type, CaLPAT2-RNAi could significantly reduce linoleic and linolenic acid content, simultaneously increasing erucic acid content. Systematic metabolism engineering was then carried out to further study CaLPAT2-RNAi, combined with the overexpression of *Brassica napus fatty acid elongase* (*BnFAE*), *Limnanthes douglasii LPAT* (*LdLPAT*), and RNAi of endogenous *fatty acid desaturase 2* (*CaFAD2-RNAi*). Oil composition analysis on the transformants' seeds showed that (a) with *CaFAD2-RNAi*, PUFA content could be dramatically decreased, in comparison with *BnFAE* + *LdLPAT* + *CaFAD2-RNAi*, and *BnFAE* + *LdLPAT* + *CaFAD2-RNAi* + *CaLPAT2-RNAi* seeds showed lower linolenic acid content; (b) *BnFAE* + *LdLPAT* + *CaFAD2-RNAi* + *CaLPAT2-RNAi* could increase the erucic acid content in crambe seed oil from less than 66.6% to 71.6%, whereas the highest erucic acid content of *BnFAE* + *LdLPAT* + *CaFAD2-RNAi* was 79.2%; (c) although the four-gene combination could not increase the erucic acid content of seed oil to a higher level than the others, it led to increased carbon resource deposited into C22:1 and C18:1 moieties and lower PUFA. Summarily, the present research indicates that suppression of *LPAT2* is a new, promising strategy for seed-oil biosynthesis pathway engineering, which would increase the value of crambe oil.

KEYWORDS

Crambe abyssinica, lysophosphatidic acid acyltransferase, metabolism pathway regulation, oil, salt land improvement

1 | INTRODUCTION

The oil stored in plant seeds (mostly as triacylglycerol [TAG]) is not only a crucial nutrition for seedling growth (Carlsson, 2009; Chapman & Ohlrogge, 2012; Gurr et al., 1974) but also an essential renewable resource for humans. Oil seed crop cultivation is an important part of global agriculture. Erucic acid (omega-9 C22:1 fatty acid) is a very important lipid compound produced by plants. Erucic acid and its derivatives, such as erucamide, behenic acid, or alcohol, have wide industrial uses as, for example, surfactants, lubricants, and pour point

depressants (Rudloff & Wang, 2011; Vargas-Lopez, Wiesenborn, Tostenson, & Cihacek, 1999). Erucic acid only occurs in the seed oil of the plant families *Brassicaceae* and *Tropaeolaceae*. The oil seed plant *Crambe abyssinica* (crambe), which is of *Brassicaceae* family and also known as Abyssinian mustard, Abyssinian kale, colewart, or datran, is a new crop usually cultivated to produce high erucic acid plant oil and other valuable chemical compounds for industry (Bruun & Matchett, 1963; White & Higgins, 1966). Crambe is a low-input crop and grows well on all soil types from heavy clays to light sands and is tolerant to salinity and several other kinds of abio-stress (Falasca,

Flores, Lamas, Carballo, & Anschau, 2010; Fowler, 1991; Vasconcelos, Chaves, Souza, Gheyi, & Fernandes, 2015). It could be cultivated on saline soil and tolerant to salty water irrigation. Therefore, it can be used as an alternative crop in marginal areas near coastlines for salt land improvement (Falasca et al., 2010). Increasing the erucic content in crambe seed oil and decreasing the polyunsaturated fatty acids (PUFAs) by breeding or genetic engineering could intensively elevate the economic value of this crop (Falasca et al., 2010).

Erucic acid is produced by ketoacyl-CoA synthase (FAE) of cytosol fatty acid elongase complex from C18:1 CoA in the developing seed embryo of *Brassicaceae* plants. Subsequently, erucic CoA is integrated into the glycerol backbone by the acyltransferase of the Kennedy pathway that takes place in the endoplasmic reticulum (Kennedy, 1961; Murphy & Vance, 1999). In the first two steps, glycerol-3-phosphate is acylated by glycerol-3-phosphate acyltransferase into lysophosphatidic acid (Murata & Tasaka, 1997; Zheng & Zou, 2001), and it is then acylated further by lysophosphatidic acid acyltransferase (LPAT) into phosphatidic acid (Khaik-Cheang and Huang, 1989; Kim, Li, & Huang, 2005; Soupene, Fyrst, & Kuypers, 2007). This is followed by dephosphorylation of phosphatidic acid catalysed by phosphatidate phosphatase to release diacylglycerol (DAG; Kocsis, Weselake, Eng, Furukawa-Stoffer, & Pomeroy, 1996). The final acylation of DAG, catalysed by diacylglycerol acyltransferase (DGAT; Cases et al., 1998; Routaboula, Benningb, Bechtoldc, Cabochea, & Lepinieca, 1999), generates TAG that is transferred to oil bodies for storage (Huang, 1996; Murphy & Vance, 1999). The catalysis of C18:1 CoA to PUFA (i.e., linoleic acid and linolenic acid) by FAD is a well-known substrate-competition to erucic acid biosynthesis (Cheng et al., 2013; Jadhav et al., 2005; Okuley et al., 1994).

High erucic acid vegetable oil is that which contains over 55% erucic acid, and this is a valuable feedstock for the chemical industry. Nowadays, most high erucic acid vegetable oil supplied to the chemical industry comes from high erucic acid rapeseed (HEAR). The cultivation of HEAR, however, is problematic because of the risk of contaminating food quality rapeseed (Canola) by either inadvertent mixing or cross-pollination. Therefore, HEAR cultivars are cultivated in Europe (about 40,000 hectares in 2006/2007) and in the USA and Canada as an identity-preserved crop, because oil from HEAR (high erucic acid rapeseed oil, HERO) should not enter the food chain. The upper limit of erucic acid in food oil rapeseed has been set to 5% in the EU (Council Directive 76/621/EEC), although the risk of erucic acid consumption was only inferred from effects on rats and in humans, no epidemiological evidence is available that erucic acid consumption leads to an increase in cardiovascular diseases (Hu, Sullivan-Gilbert, Gupta, & Thompson, 2006; Schierholt, Becker, & Ecke, 2000). For infant formulas, EC Directive 2006/141/EC (2006) states a limit of 1% erucic acid of total fatty acids. Because of the health risk, another member of the *Brassicaceae* family, *C. abyssinica* (crambe) is considered as a candidate to substitute HEAR for high erucic acid vegetable oil production (Bruun & Matchett, 1963; Rudloff & Wang, 2011). The main advantages of crambe, with regards to the risk of erucic acid vegetable oil entering the food chain are (a) identity preservation is easy as it is morphologically very distinct from rapeseed, both as a crop and as seed; (b) it does not outcross with rapeseed as HEAR can with Canola. Technically, crambe also has advantages over HEAR as it has a higher erucic acid content (59–65%) in its seed oil than HEAR (50–55%); and (c) the oil contains relatively less

PUFAs, which could benefit the downstream process of erucic acid isolation.

As a crop of bio-based economic value, crambe could become even more attractive to farmers and end users if crambe seed oil fatty acid composition could be optimized further, for instance, increasing erucic acid content, decreasing PUFA content, and so forth. The content of erucic acid in the seed oil of crambe can be up to 65% depending on growth conditions and cultivars and has almost reached its maximum. Enzymatic analysis on *Brassicaceae* developing seeds shows that the LPAT2 enzyme in charge of catalysing the incorporating of fatty acids into triglycerides on the sn-2 position cannot use erucic acid as a substrate (Kuo & Gardner, 2002). This means erucic acid can only be incorporated on the sn-1 and sn-3 positions of glycerol and not on the sn-2 position of triglycerides, which limits the potential erucic acid content in *Brassicaceae* crop seed oil to 66.7% (2/3). Because of this, genetic modification strategies have been developed to enable incorporating erucic acid on the sn-2 position by introducing a *Limnanthes douglasii* LPAT (LdLPAT) that takes erucic acid as a substrate for lysophosphatidate sn-2 acylation (Cao, Khaik-Cheang, & Huang, 1990; Lassner, Levering, Davies, & Knutzon, 1995; Laurent & Huang, 1992). Previous research on *Brassica napus* (Han et al., 2001) and crambe (Li et al., 2012) shows that erucic acid levels could be elevated by introducing the *LdLPAT* gene, *B. napus* fatty acid elongase (ketoacyl-CoA synthase, *BnFAE*), which is in charge of prolonging oleic acid into erucic acid (Mietkiewska, Brost, Giblin, Barton, & Taylor, 2007), and the RNAi sequence targeted at native fatty acid desaturase 2 (*CaFAD2-RNAi*), which is in charge of desaturating oleic acid into linoleic acid and linolenic acid (Cheng et al., 2013). In the present study, endogenous LPAT (*CaLPAT2*) suppression was engaged into the new approach with the aim of manipulating crambe seed oil composition by genetic modification. First, an RNA interference construct (*CaLPAT2-RNAi*; Wesley et al., 2003) was developed to target a conserved sequence of LPAT2 of crambe as well as that of other *Brassicaceae* species. *CaLPAT2-RNAi* was then combined with *BnFAE*, *LdLPAT*, and *CaFAD2-RNAi* into one vector that was engaged to regulate oil compounds in crambe seeds. Simultaneously, vectors of *BnFAE* + *LdLPAT* and *BnFAE* + *LdLPAT* + *CaFAD2-RNAi* were also transformed into crambe for comparison.

2 | MATERIALS AND METHODS

2.1 | Vectors used in present approach

As shown in Figure 1, four binary vectors were used in this study. The vector *CaLPAT-RNAi* was composed of the RNA interference gene against *CaLPAT2*, which was controlled by the 35S promoter and terminator. The *BnFAE* + *LdLPAT* and *BnFAE* + *LdLPAT* + *CaFAD2-RNAi* vectors were acquired from the Unit of Botany, Aachen University of Technology. The *BnFAE* + *LdLPAT* vector carries two genes, *LdLPAT* and *BnFAE* and *BnFAE* + *LdLPAT* + *CaFAD2-RNAi* contains *LdLPAT*, *BnFAE*, and *CaFAD2-RNAi*. The *BnFAE* + *LdLPAT* + *CaFAD2-RNAi* + *CaLPAT2-RNAi* construct containing *LdLPAT*, *BnFAE*, *CaFAD2-RNAi*, and *CaLPAT2-RNAi* was acquired after the molecular modification of

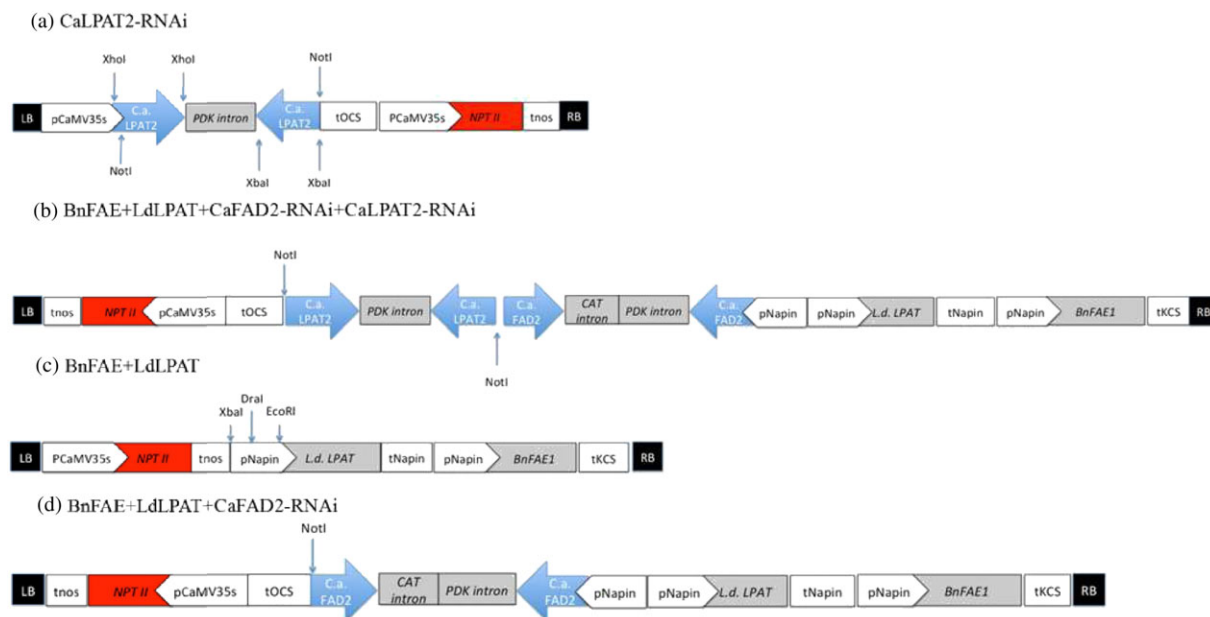


FIGURE 1 The functional genes contained by the four vectors used in the present research: Four vectors were used in this study. All have *NPTII* as the selectable marker for plant transformation. In *CaLPAT2-RNAi* vector (a) the *CaLPAT2 RNAi* was controlled by pCaMV35s promoter. In the other vectors, genes adjusting plant seed oil composition in BnFAE + LdLPAT + CaFAD2-RNAi + *CaLPAT2-RNAi* (b), BnFAE + LdLPAT (c), and BnFAE + LdLPAT + CaFAD2-RNAi (d) were ruled by *Brassica napus* Napin promoter (pNapin). LB = left border; RB = right border; tnos = NOS terminator; tOCS = OCS terminator; tKCS = KCS terminator) [Colour figure can be viewed at wileyonlinelibrary.com]

BnFAE + LdLPAT + CaFAD2-RNAi. Functional genes in BnFAE + LdLPAT, BnFAE + LdLPAT + CaFAD2-RNAi, and BnFAE + LdLPAT + CaFAD2-RNAi + *CaLPAT2-RNAi* were controlled by the Napin promoter. All of those vectors contained the *NPTII* gene as a selectable marker. For inoculating plants, *CaLPAT-RNAi*, BnFAE + LdLPAT + CaFAD2-RNAi + *CaLPAT2-RNAi*, and BnFAE + LdLPAT + CaFAD2-RNAi were transformed into agrobacterium strain AGL1, whereas BnFAE + LdLPAT was introduced in AGL0 (Lazo, Stein, & Ludwig, 1991).

2.2 | Construction of *CaLPAT-RNAi* vector

The target coding region of *CaLPAT2* was cloned from cDNA of developing seeds (20 days after pollination) of *C. abyssinica* cv. Galactica by polymerase chain reaction (PCR) using specific primers (forward primer: CACC-GGGTAAAGAACATGCTCTTG and reverse primer: AGTTTA GCCTCAGTAAATCG, designed according to the cDNA sequence of crambe LPAT2 acquired from NCBI, GI: 124378834; Figure 2). The amplicon was verified by sequencing and then ligated into donor vector pENTR/D-TOPO using the Gateway® BP Clonase™ II enzyme mix (Invitrogen) to construct entry vector pENTR/D-TOPO::CaLPAT2. The target fragment in the entry vector was subsequently exchanged into Gateway destination vector pHellsgate 8 (Wesley et al., 2003) using the Gateway LR® Clonase™ II enzyme mix (Invitrogen). Both constructs were transformed into *Escherichia coli* (strain: TOP10, Invitrogen, Cat. no. 4040-50) for amplification. The transformants of pENTR/D-TOPO::CaLPAT2 were checked by colony PCR analysis using primers M13 forward and reverse (GTAAACGACGGCCAG and CAGG AAACAGCTATGAC). The transformants of pHellsgate *CaLPAT2-RNAi* were checked by restrictions of XbaI and XhoI, respectively. Finally, the accurate pHellsgate-*CaLPAT2-RNAi* construct was transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation.

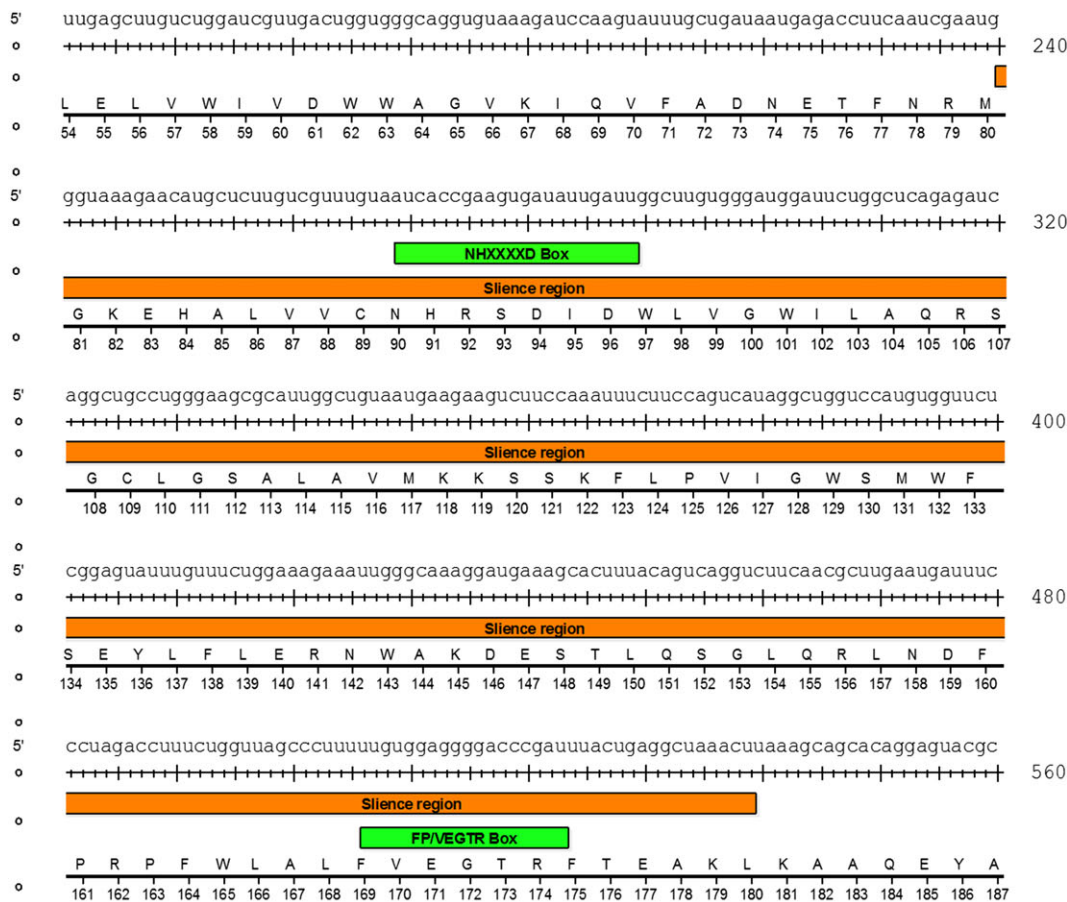
2.3 | Modifying BnFAE + LdLPAT + CaFAD2-RNAi into BnFAE + LdLPAT + CaFAD2-RNAi + *CaLPAT2-RNAi*

As shown in Figure 1, in the DNA molecule of BnFAE + LdLPAT + CaFAD2-RNAi, there is only one restriction site of *NotI* restriction enzyme in the connection region of CaFAD2-RNAi palindromic sequence to the Napin promoter; and in the DNA molecule of pHellsgate *CaLPAT-RNAi*, there are two *NotI* restriction sites between which the palindromic sequence of *CaLPAT-RNAi* is located.

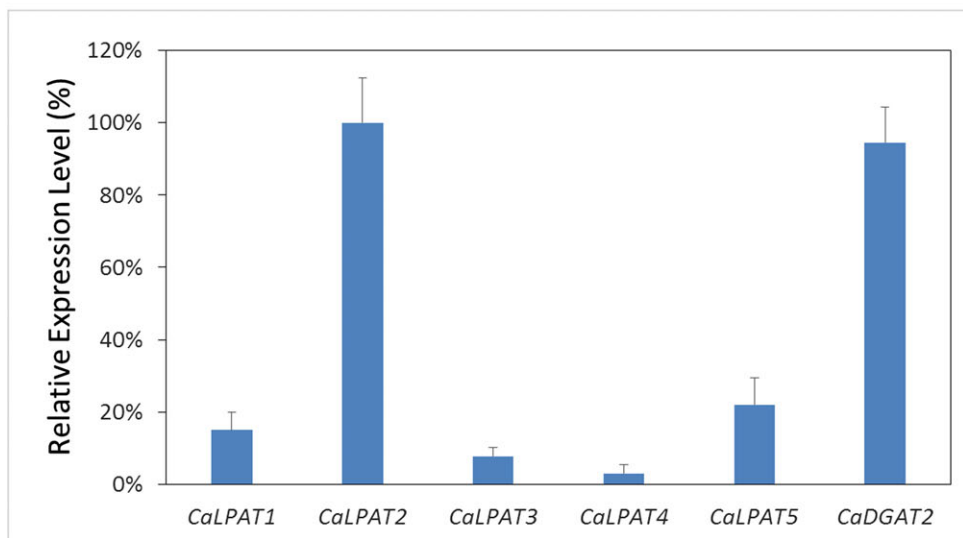
The pHellsgate *CaLPAT-RNAi* vector DNA was digested by *NotI*. The digested DNA fragments were then separated by electrophoresis with 0.8% agarose gel, by which the desired palindromic fragment (364 bp) was separated. After reclaiming the fragment from the gel, it was ligated with the linear BnFAE + LdLPAT + CaFAD2-RNAi molecule cut by *NotI*. From the modification, the BnFAE + LdLPAT + CaFAD2-RNAi + *CaLPAT2-RNAi* vector was constructed. In this new vector, there are two palindrome structures (*CaFAD2-RNAi* and *CaLPAT2-RNAi*) side-by-side, sharing the same promoter (Napin) and terminator (tOCS). The ligation product was transformed into *E. coli* competent cells (strain: TOP10 Chemical, Invitrogen) by electroporation. Candidate colonies' plasmid DNA was isolated and digested again by *NotI*. Those giving a unique band of the original size (364 bp) were chosen for transforming agrobacterium strain AGL1.

2.4 | Plant transformation

C. abyssinica seeds (cv. Galactica, harvested July 2009 from a seed production field in Wageningen, The Netherlands) were germinated to obtain cotyledonary node explants as starting material for



(a)



(b)

FIGURE 2 (a) The target sequence picked up for *Crambe abyssinica* endogenous *LPAT2* RNAi: A segment of mRNA of *C. abyssinica* *LPAT2* and the corresponding peptide is displayed in the chart. The orange bar marks the whole sequence (360 BP) later incorporated into the RNAi vector, and the two green bars show two conserved regions, the NHXXXD box and FP/VEGTR box. (b) The expression level of Arabidopsis *LAPT* 1–5 homologous genes of crabwe in the developing seeds: The expression level of Arabidopsis *LPAT* 1–5 homologous genes of *C. abyssinica* in the developing seeds 20 days after flowering was assayed by quantitative polymerase chain reaction. The results showed that *LPAT2* had the strongest expression that was 7 to 20 folds higher than the others. The expression level of Arabidopsis *diacylglycerol acyltransferase 2* homologues in the same period was as same as *CaLPAT2*. WT = wild type [Colour figure can be viewed at wileyonlinelibrary.com]

transformation and regeneration. The in vitro material was cultivated in growth chambers with a photoperiod of 16 hr with a light intensity of 33 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a temperature of 24 °C.

The transformation and selection for GM events of *C. abyssinica* in this chapter were performed as we described previously (Qi et al., 2014).

Arabidopsis thaliana plants (Accession, Columbia) were grown in the greenhouse or in the climate chamber with the following settings: 22/19 °C with 70% relative humidity and 16/8 hr day/night periods, respectively. If the light intensity dropped below 150 W·m⁻², it was compensated with an extra 100 W·m⁻² supplemental light. *A. tumefaciens* with target pHellsgate CaLPAT2-RNAi construct was used to transform *Arabidopsis* plants using the floral dip method (Clough & Bent, 1998). Kanamycin selection of positive transformants was performed (Harrison et al., 2006).

2.5 | Identifying transformants

2.5.1 | Southern blotting

From BnFAE + LdLPAT T0 candidate transformants genomic DNA was isolated from the leaf material of in vitro plants with the method described by Aldrich and Cullis (1993) but with 1% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer. For each transformant, the DNA isolation was performed 3 times independently for each candidate. For each DNA isolation, 1 g fresh leaf material was used. Three pieces of DNA samples (40 µg per piece) from each candidate transformant were digested by EcoRI, Dral, and XbaI. The T-DNA region of BnFAE + LdLPAT is shown in Figure 1. The *NPTII* gene is located near the left border. The nearest restriction sites of EcoRI, Dral, and XbaI to the left border are also marked in Figure 1. For copy number determination, a probe (516 bp) was designed based on the sequence of the *NPTII* gene and was labelled with [³²P]ATP. The digested DNA samples were fractionated on a 0.8% (w/v) agarose gel and transferred to Hybond N+ membrane (Amersham Biosciences, UK) according to the manufacturer's recommendations. The membrane was hybridized at 65 °C overnight with 20 ng of the *nptII* probe and washed for 2 × 30 min with 0.1 × saline-sodium citrate buffer, 0.1% (w/v) SDS at 65 °C. The DNA gel blots were exposed to a phosphorimager screen and subsequently scanned into a Bioimager device (Fujix BAS2000).

The primers of probe amplification were TCCAAGATGTAGCATC AAGAATCC (forward) and TGGTTTCGATCCACTTCTTAC (reverse).

PCR

Genomic DNA was isolated from young leaves of each candidate T0 plant with the same method as used for the Southern blotting. The

NPTII primers are used to identify the transgenic nature of candidate materials. The *virG* primers are used to amplify the *virG* gene from the vector backbone to test for its presence in the material. If the result of *virG* is positive, then the material cannot be counted as a GM candidate or moved into soil for seed ripening. They should remain in the medium with cefotaxime and timentin until such time that there are no longer positive PCR results for *virG*.

The primer sequences for *NPTII* were forward 5'- TGGGCACAA CAGACAATCGGCTGC-3' and reverse 5'-TGCGAATCGGGAGCGG CGATACCG-3' and for *virG*, forward 5'-GCCGGGGCGAGACCAT AGG-3' and reverse 5'-CGCACGCGCAAGGCAACC-3'.

qPCR

Two pairs of primers specific to the crambe *LPAT2* genes were developed based on the cDNA sequences in *C. abyssinica* (GI: 124378834), and two pairs of primers of *Arabidopsis LPAT2* gene were developed on the cDNA sequences of *AtLPAT2* (GI:145339616). The gene β -actin 2 (GI: 20465834) was used as a reference gene. These primers used in real-time PCR are listed in Table 1. The primers used to evaluate the expressions of *Arabidopsis LPAT1*, 3, 4, 5, and *DGAT2* homologous genes in crambe were the same as previous reports (Kim et al., 2005; Li, Yu, & Hildebrand, 2010). To develop crambe seed RNA isolation, total RNA was extracted from bulked seeds of T0 plants (10 seeds per plant, 20 days after flowering [DAF]) with an RNeasy Plant Mini Kits (Qiagen, Germany) according to the manufacturer's instructions. For the leaf RNA isolation of crambe and *Arabidopsis*, total RNA was extracted from 0.5 g fresh leaf material from the greenhouse plant with the same kit. The isolated RNA was treated with RNase-free TURBO DNase (Ambion, USA) to remove residual genomic DNA. First-strand cDNA was synthesized in 20 µl from 1 µg of total RNA with an iScript™ cDNA Synthesis Kit (Bio-rad, USA). The cDNA samples were 20× diluted and used as templates for real-time PCR. The PCR reaction contains 2 µl templates, 5 µl SYBR Green Super Mix (Bio-rad, USA), and 1 µl of each of the forward and reverse primers (3 µM), giving 10 µl. Cycling conditions were 1 cycle at 95 °C for 3 min followed by 30 cycles at 95 °C for 10 s, 60 °C for 1 min, then a final melt step from 65 °C to 95 °C ramp with 0.5 °C increments per cycle to monitor specificity. PCR reactions were performed in triplicate. The expression of each replicate was normalized by the reference gene, β -actin 2, which has shown to be stably

TABLE 1 The quantitative polymerase chain reaction primers for testing *LPAT2* expression levels in *Arabidopsis* leaf, crambe leaf, and crambe developing seeds

| Target gene | Primer name | Sequence |
|-------------------------------------------------------------|-------------|----------------------------|
| <i>Arabidopsis LPAT2</i> (GI:145339616) | AtLPAT2_F1 | GGT GGT TGC AGA AAC CTT GT |
| | AtLPAT2_R1 | AGC ATG TTC TTT GCC CAT T |
| | AtLPAT2_F2 | GGT GGT TGC AGA AAC CTT GT |
| | AtLPAT2_R2 | GAG CAT GTT CTT TGC CCA TT |
| <i>Arabidopsis β-actin 2</i> (GI:20465834) | AtActin2_F | GATGGAGACCTCGAAAACCA |
| | AtActin2_R | AAAAGGACTTCTGGGCACCT |
| <i>Crambe LPAT2</i> GI:124378834 | CaLPAT2_F1 | CGC ATT GGC TGT AAT GAA GA |
| | CaLPAT2_F2 | CCA GTC ATA GGC TGG TCC AT |
| | CaLPAT2_R1 | TTC ATC CTT TGC CCA ATT TC |
| | CaLPAT2_R2 | CCA CAA AAA GGG CTA ACC AG |
| <i>Crambe FAD2</i> | CaFAD2-F1 | CCGTGAACGTCTCCAGATAT |
| | CaFAD2-R1 | CGTTGACTATCAGAAGCCGA |

expressed in crambe seedlings under arsenate stress and in various *B. napus* cultivars (Hu et al., 2009; Paulose, Kandasamy, & Dhankher, 2010). The relative expression level of each replicate was calculated according to the comparative CT method (User Bulletin No. 2, ABI PRISM 7700 Sequence Detection System, December 1997; Perkin-Elmer, Applied Biosystems). The mean of three replicates represents the relative expression level of a line.

2.5.2 | Gas chromatography analysis

Gas chromatography (GC) was engaged to analyse the oil composition of crambe seed. The wild type (WT) seeds were acquired from the control plant through tissue culture together with the transformed and grew in the same conditions. An individual seed was crushed in a 1.5 ml Eppendorf tube with 600 μ l of hexane to extract the oleic compositions (at room temperature). After centrifuging at 18,000 g for 5 min, 200 μ l of the hexane supernatant was taken into the GC vial for triacylglycerol analysis by the high temperature column. Subsequently, 40 μ l methanol (with 5 M KOH) was added into the Eppendorf tube, and the closed tube was incubated at 60 °C with shaking to hydrolyse the TAG to obtain the free fatty acids and to methylate the fatty acids to fatty acid methyl esters (FAME). The FAME composition was determined using GC (column/DB-23, Agilent). A 1- μ l sample was injected into the GC with FID with a split ratio of 1:20. A temperature gradient was used starting with 10 min at 180 °C, a temperature increase to 240 °C for 8 min, and 7 min at 240 °C. Identification of FAME was based on retention time of standards and checked by separate GC-MS analysis using the same column and temperature profile. The relative amounts of different FAME were determined as the relative peak area of the components in the total peak area of FAME. Table 2 illustrates the quantity of seeds that were put into the GC FAME assay.

3 | RESULTS

3.1 | Cloning the conserved sequence of CaLPAT2 to construct the RNAi vector

The conserved region encoding the NHXXXXD box, FP/VEGTR box, and the connecting region in *CaLPAT2* mRNA was chosen as the target of RNAi (Figure 2a; Murphy, 2009). The expression levels of the homologues of Arabidopsis *LPAT1-5* in *C. abyssinica* (*CaLPAT*) were assayed by qPCR (Figure 2b). In comparison with the other *CaLPATs*,

TABLE 2 The seeds used for FAME GC analysis

| Generation | Vector | Seed family Nr. | Seed Nr. |
|------------|--------------------------------------------|-----------------|----------|
| | Wild type | 3 | 60 |
| T1 | CaLPAT2-RNAi | 2 | 60 |
| T1 | BnFAE + LdLPAT | 3 | 100 |
| T1 | BnFAE + LdLPAT + CaFAD2-RNAi | 5 | 120 |
| T1 | BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2RNAi | 4 | 120 |

Note. Seed family Nr = number of seed families chosen for the assay; Seed Nr = number of seeds from those families analysed by FAME GC. FAME = fatty acid methyl esters; GC = gas chromatography.

CaLPAT2 had the highest expression level that was 7 to 20 folds higher than the others. The expression level of Arabidopsis *diacylglycerol acyltransferase 2* homologues (*CaDGAT2*) in the same period was as same as *CaLPAT2*. The sequence was cloned by PCR from the cDNA of crambe seed 20 DAF. It was integrated into clone/entry vector pENTR/D-TOPO, of which the success was verified by sequencing. Finally, the target fragment in pENTR/D-TOPO was recombined into the empty pHellsgate 8 vector, to build the *CaLPAT*-RNAi. The desired RNAi vectors were identified by restriction of XbaI and XhoI, respectively. As shown in Supplementary Figure 1, both of the digestions gave the bands of expected sizes, which indicated the successful construction of the *CaLPAT2*-RNAi vector.

3.2 | Transformation of Arabidopsis and crambe with CaLPAT-RNAi

Arabidopsis (Accession, Columbia) and crambe were transformed with construct *CaLPAT2*-RNAi using *Agrobacterium*. There were five independent T0 crambe lines and 20 T1 Arabidopsis lines acquired after Kanamycin selection and confirmed by PCR.

The expression of endogenous *LPAT2* in the transgenic plants was downregulated because of the RNAi, which was confirmed by the qPCR. Among the 20 T1 lines of Arabidopsis, the most efficient RNAi effect is shown in Figure 3a. The *LPAT2* expression level in the GM plant leaf tissue was about 20-fold lower than the wild type (Student's *t* test: $p < .01$). The phenotypes of shorter silique and seed abortion were observed in parallel with the low expression level of *LPAT2* in the T1 Arabidopsis plants (Figure 4).

Compared with the WT, the T0 crambe with the lowest *LPAT2* expression was around 50% decreased in leaf tissue (Student's *t* test: $p < .01$), and 80% downregulated in developing seeds (Figure 3b). Crambe has only one seed per pod, so abortion of a seed results in empty pods. No difference in seed abortion or seed size between WT plant and the *CaLPAT*-RNAi transgenic plant was found in this experiment.

3.3 | Identification of transgenic events of BnFAE + LdLPAT, BnFAE + LdLPAT + CaFAD2-RNAi, and BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi

The vectors BnFAE + LdLPAT, BnFAE + LdLPAT + CaFAD2-RNAi, and BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi were transformed into *C. abyssinica*. After selection by Kanamycin and verification by PCR using primers of *NPTII* and *VirG*, there were three independent T0 lines of BnFAE + LdLPAT, eight of BnFAE + LdLPAT + CaFAD2-RNAi, and four of BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi obtained. The T-DNA insertion number of BnFAE + LdLPAT T0 lines was verified by Southern blotting (Figure 5). Of the BnFAE + LdLPAT independent transformants, one had two T-DNA inserts, one had three (or four) T-DNA inserts, and one had more than six T-DNA inserts.

The expression of *CaFAD2* and *CaLPAT2* was assayed by qPCR in developing seeds (20 DAF) of WT, a T0 line of BnFAE + LdLPAT + CaFAD2-RNAi and two T0 lines of BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi. In comparison

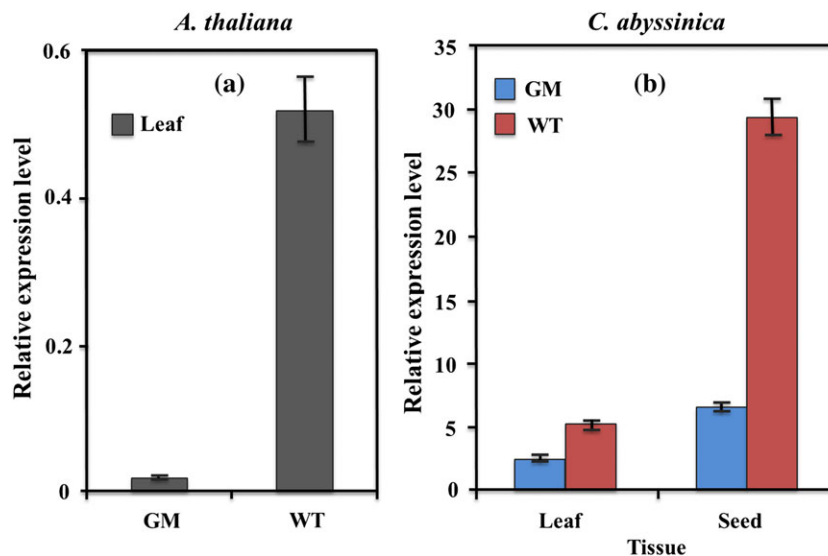


FIGURE 3 Quantitative polymerase chain reaction (qPCR) tests on CaLPAT2-RNAi GM plant materials of *Arabidopsis* (a) and crambe (b): Endogenous *LPAT2* expression levels of the leaf materials from T1 *Arabidopsis* and T0 crambe plants and developing T1 crambe seeds were analysed by qPCR. The GM lines indicating the highest decrease levels are shown in the charts. The column is the average of repeats, and the bar is standard deviation. The qPCR result of transgene *Arabidopsis* and crambe with the most efficient RNAi effect is demonstrated in the charts. In comparison with the wild type, the *LPAT2* expression of transgene *Arabidopsis* and crambe was suppressed into significantly lower levels in different tissues (according to Student's *t* test analysis) [Colour figure can be viewed at wileyonlinelibrary.com]

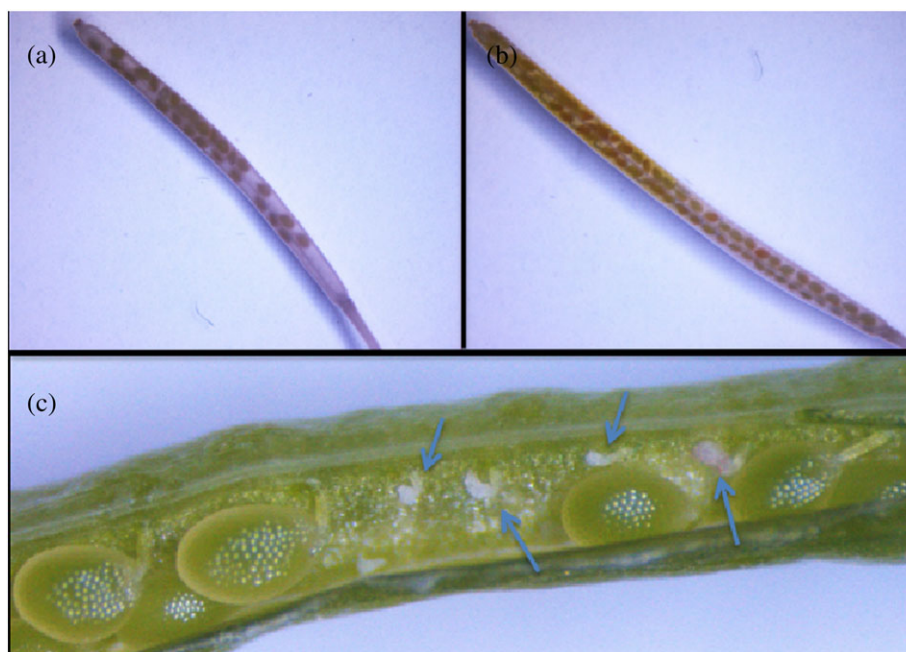


FIGURE 4 The phenotype of *LPAT2* knockdown in *Arabidopsis* by RNAi: The T1 plant of *Arabidopsis* CaLPAT2-RNAi transformed showed shorter siliqua (a) than that of the wild type (b). Inside the developing siliqua, a fraction of the developing seeds were aborted (c, marked by arrows) [Colour figure can be viewed at wileyonlinelibrary.com]

to the WT, the expression of *CaFAD2* in the transgenic developing seeds of BnFAE + LdLPAT + CaFAD2-RNAi and BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi was about fourfold to fivefold downregulated

(Figure 6a). The expression of *CaLPAT2* was only around fourfold decreased in the developing seeds from BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi (Figure 6b).

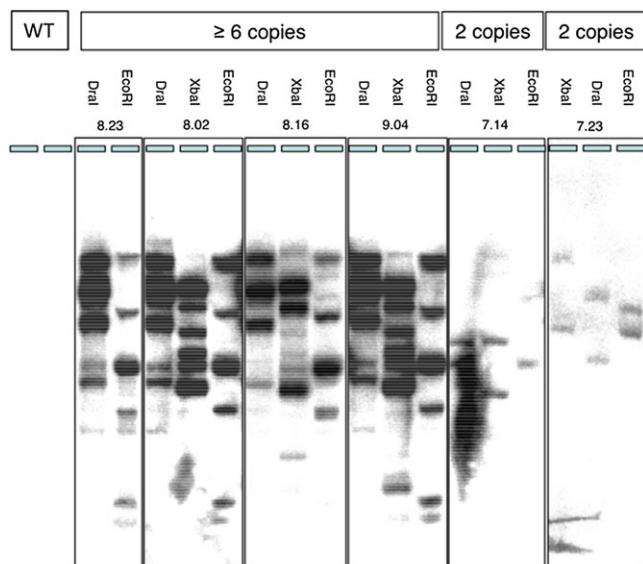


FIGURE 5 Southern blotting analysis of T0 crambe plant of BnFAE + LdLPAT transformation: The probe was designed according to *NPTII* gene, and three restriction enzymes (DraI, XbaI, and EcoRI) were used for DNA digestion. The southern blotting result demonstrated that one line (ID: 9.04) had more than six T-DNA insertions, line 7.23 had two T-DNA insertions, and the T-DNA insertion number of 7.14 was two or three. WT = wild type [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Variation of PUFA content of the T1 seed oil

According to the crambe seed oil fatty-acid composition, its PUFA consists mainly of linoleic fatty acid (C18:2) and linolenic fatty acid (C18:3). In the WT seed oil, the C18:2 content varied from 6.7% to 11.2%. The CaLPAT2-RNAi and the CaFAD2-RNAi contained vectors that could downregulate linoleic acid content in seed oil significantly, but the transgenic of BnFAE + LdLPAT had the same C18:2 content as the WT, as showed in Figure 7a. Meanwhile, the linolenic acid content of the transgenic seed was generally significantly decreased. The four-gene combination had the most severe effect, and the linolenic acid content was less than half of the WT. The CaLPAT-RNAi downregulated the C18:3 content to a significantly lower level, similar to BnFAE + LdLPAT + CaFAD2-RNAi.

3.5 | The variation of erucic acids in the T1 seed oil of transformants

Based on the qPCR results, seeds from the pHellsgate CaLPAT2-RNAi T0 plant with the most significant CaLPAT2 suppression were selected for GC analysis. Compared with wild type, the T1 seed oil had a generally stable and significantly higher level of erucic acid content: 64.5% on average and ranging from 63.1% to 66.3%. There were four T1 seed families of BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi assayed. Erucic acid content in single seed oil varied on a large scale. Single seeds with more than 70% erucic acid in its oil were found in three families; the highest value of single seed was 71.6%. However, the highest erucic acid content, as much as 79.2%, was found in the transgene seeds of BnFAE + LdLPAT + CaFAD2-RNAi.

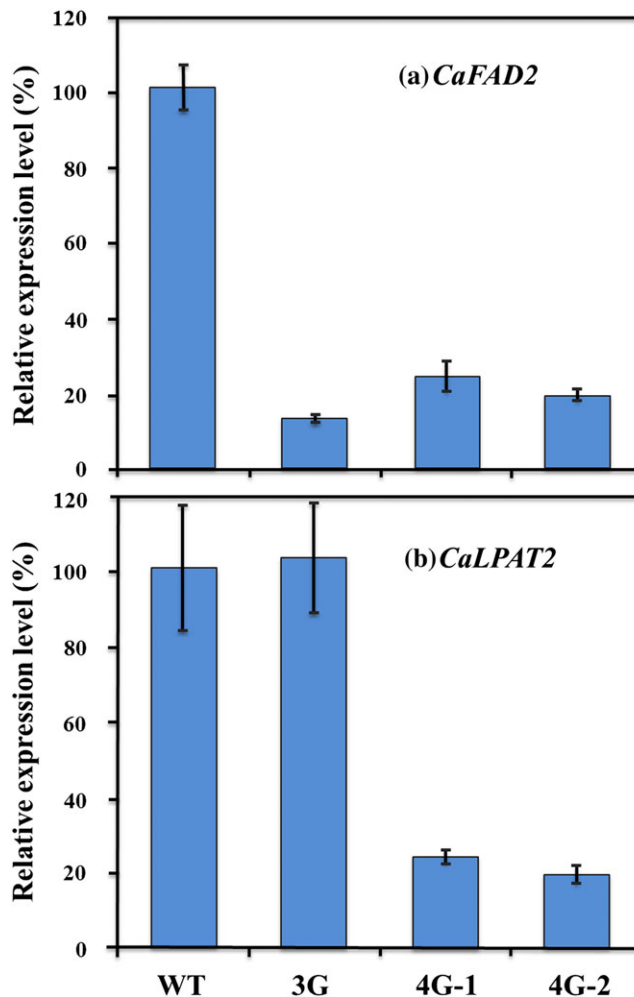


FIGURE 6 Quantitative polymerase chain reaction analysis of the developing GM seeds of BnFAE + LdLPAT + CaFAD2-RNAi and BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi transformations: The T1 developing seeds of 20 days after flowering were used for this assay. Two T0 BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi plants (4G-1 and 4G-2) and one T0 BnFAE + LdLPAT + CaFAD2-RNAi plant (3G) were selected for the test. The wild type (WT) seeds were acquired from the control plant through tissue culture together with the transformed. Panel (a) shows the expression level of CaFAD2; Panel (b) shows the expression level of CaLPAT2 [Colour figure can be viewed at wileyonlinelibrary.com]

Meanwhile, among the transgene seeds of BnFAE + LdLPAT, none showed erucic acid content higher than 66.4%. One-way analysis of variance showed that the erucic acid content in the seeds of CaLPAT2-RNAi and the four-gene and three-gene constructs were significantly higher than those of the WT and two-gene vector. The erucic acid content distribution is shown by the box-blot in Figure 7c.

Previous research showed that introducing *BnFAE* into the crambe genome might cause a possible cosuppression phenotype of which the oleic content became ultra-high but the erucic acid low (Li et al., 2012). A similar phenomenon was also discovered in present research. To avoid the unexpected influence of evaluating the trait associated with CaLPAT2-RNAi, the data of seeds with suppressed erucic acid content lower than the wild type were excluded and not shown here.

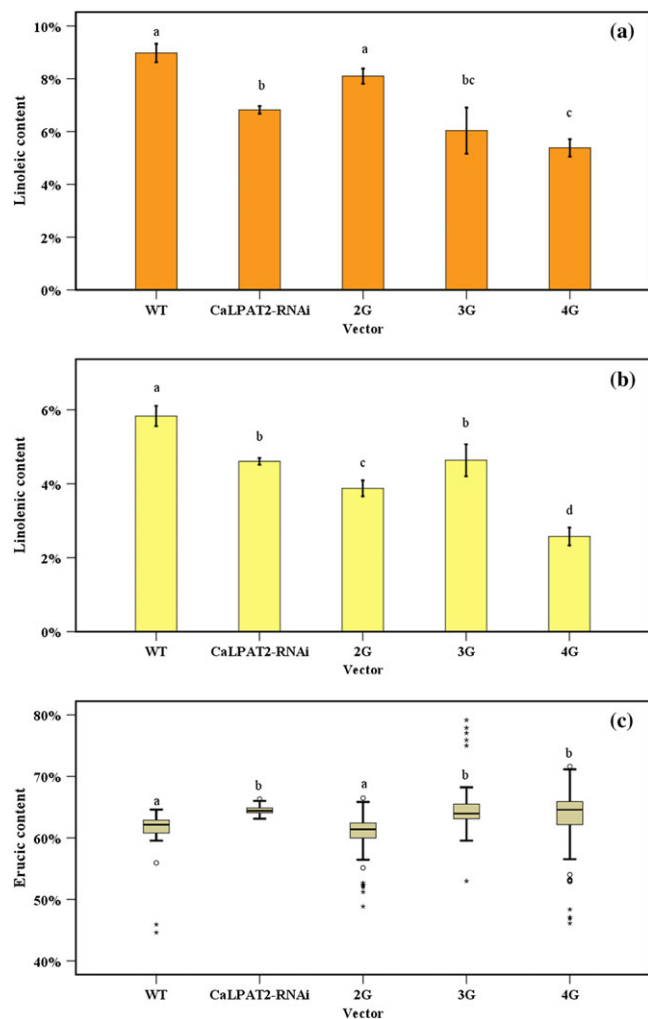


FIGURE 7 Linoleic, linolenic, and erucic acid content of T1 seeds: In crambe seed oil, the main composition of PUFA were linoleic and linolenic acid. They were acknowledged as the major competitors of erucic acid in the fatty acid biosynthesis and accumulation pathways. Linoleic, linolenic, and erucic acid content of T1 seeds are demonstrated in Panels (a), (b), and (c). In Panels (a) and (b), the column is the mean, and the bars are standard deviation; (c) is a boxplot: The letters on the top of bars indicate the significant differences (analysis of variance: Tukey). All of the transgene seeds had lower PUFA accumulated than the wild type (WT). 2G: BnFAE + LdLPAT; 3G: BnFAE + LdLPAT + CaFAD2-RNAi; 4G: BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

There are five known LPAT isoforms (LPAT1, 2, 3, 4, and 5) of Arabidopsis (Kim et al., 2005). All except LPAT1 are located on the endoplasmic reticulum. Among the genes encoding LPAT isoforms, LPAT2 always has much higher expression levels in various tissues (callus, inflorescence, leaf, root, and silique) than the others. The present research showed that, in the developing seeds, the LPAT2 homologous gene also had a higher expression level than the others. The present research also indicated that the LPAT2 expression of Arabidopsis and crambe could be suppressed efficiently by the RNAi targeting of the encoding sequence of NHXXXX box, FP/VEGTR

box, and the connecting region in between (Frentzen & Wolter, 1998). The CaLPAT2-RNAi transformed *A. thaliana* had an efficiently suppressed LPAT2 expression in leaves and the expected phenotype (shorter pods and a high proportion of seed abortion) was extremely similar to the T-DNA insert mutant (Kim et al., 2005). The transformed *C. abyssinica* also showed a significantly reduced LPAT2 expression both in leaves and developing seeds but no seed abortion or difference in seed size.

By inserting the palindrome sequence from the CaLPAT2-RNAi into the BnFAE + LdLPAT + CaFAD2-RNAi vector, the BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi vector was constructed. The qPCR analysis on endogenous CaFAD2 and CaLPAT2 gene expression demonstrated that the double-RNAi was functional. The GC analysis also indicated that the T1 seed oil had enhanced erucic acid accumulation (highest value: 71.6%) and a suppressed PUFA level. We expected the BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi transformant seed would have higher erucic acid content than the seeds of all the other transformants. However, the highest value (79.2%) in the present research was from the BnFAE + LdLPAT + CaFAD2-RNAi transformant. However, a report from Li et al. (2012) showed that transformation of BnFAE + LdLPAT could also result in ultra-high erucic acid content of more than 70%. Considering the results of the present research, the suppression of endogenous LPAT2 may not improve erucic acid content any further in crambe seed oil than the former strategy. Furthermore, Guan, Lager, Li, Stymne, and Zhu (2013) showed that in the developing crambe embryos of BnFAE + LdLPAT transgene, the erucic acid moiety was mainly locked in the phosphate choline (PC) fraction. Hence, the bottleneck was likely between PC and TAG.

The four-gene combination vector did not work as well as expected, despite the extra RNAi targeting on endogenous LPAT2. However, we did find that there were some remarkable traits concerned with CaLPAT2-RNAi. First, it was totally unexpected that CaLPAT2-RNAi alone could optimize the crambe oil composition. It is clear that downregulation of endogenous LPAT2 expression allowed increased carbon flux to go to erucic acid, and less to PUFA, even though the highest erucic acid content of the transgene seeds was 66.4%. It is not clear why this occurred. A hypothesis is that in the developing seed, when the LPAT2 was suppressed by RNAi, other functional LPATs were put in charge of TAG biosynthesis, and their substrate affinities being different to that of LPAT2 led to this fatty-acid modulation. Furthermore, the existence of CaLPAT2-RNAi together with BnFAE + LdLPAT + FAD2-RNAi also indicated some special trait in seed oil composition. In comparison with the three-gene combination, the four-gene vector had significantly restrained linolenic fatty acid accumulation. Previously, it has been reported that FAD3-RNAi could inhibit linolenic acid deposition in Arabidopsis and crambe seed oil (Li et al., 2015). But, there has been no research ever showing or implying the relationship between PUFA content and LPAT2. In present research, it was significant indeed that the four-gene transformation resulted in more carbon resource deposited into the C22:1 and C18:1 moieties (Figure 8) and lower PUFA, than the WT and the other vectors. This result demonstrates that suppression of endogenous LPAT2 is a new and promising strategy for seed-oil biosynthesis pathway engineering alternatively for improving crambe seed-oil quality and economical value of crambe cultivation.

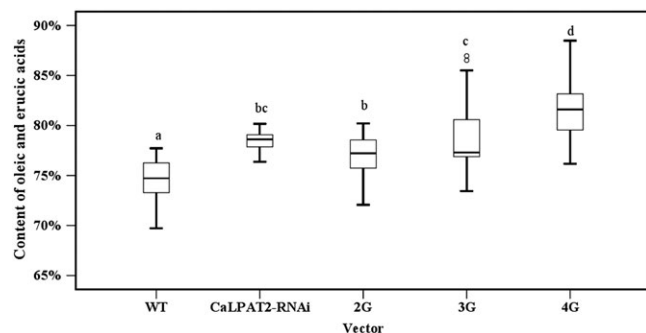


FIGURE 8 The distribution of oleic and erucic acid content: The oleic and erucic acid formed the majority of fatty acids deposited in crambe seed oil. The boxplot shows the influence from the transgene on oleic and erucic acid content in T1 seeds. The letters on the top of bars indicate the significant differences (analysis of variance: Tukey). All of the transgene seeds had lower PUFA accumulated than the wild type (WT). 2G: BnFAE + LdLPAT; 3G: BnFAE + LdLPAT + CaFAD2-RNAi; 4G: BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-RNAi

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DISCLOSURES

There is no conflict of interest regarding the paper.

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