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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

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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, colewort, 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).

Eruvic 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 lysophosphatic acid acyltransferase (LPAT) into phosphatidic acid (Khain-Chang and Huang, 1989; Kim, Li, & Huang, 2005; Soupen, Fyrst, & Kuppers, 2007). This is followed by dephosphorylation of phosphatidic acid catalysed by phosphatidate phosphatase to release diacylglycerol (DAG; Kocsis, Weselake, Eng, Furukawa-Stoffer, & Pomery, 1996). The final acylation of DAG, catalysed by diacylglycerol acyltransferase (DGAT; Cases et al., 1998; Routaboula, Benningh, 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 Linnaenthes dougassi LPAT (LdLPAT) that takes erucic acid as a substrate for lysophosphatidate sn-2 acylation (Cao, Khai-Chang, & 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 3SS 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
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, CaLPAT2-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 CaLPAT2-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-GGATTAAGACATGCTCTTG and reverse primer: AGTTTACGCCTCAGTAAATCG, 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 CAGGAAACAGCTATGAC). 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 CaLPAT2-RNAi, there are two NotI restriction sites between which the palindromic sequence of CaLPAT2-RNAi is located.

The pHellsgate CaLPAT2-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 reclaming the fragment from the gel, it was ligated into the linear BnFAE + LdLPAT + CaFAD2-RNAi + CaLPAT2-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 cotyledonal node explants as starting material for
transformation and regeneration. The in vitro material was cultivated in growth chambers with a photoperiod of 16 hr with a light intensity of 33 μmol·m²·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).

**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 LPAT1–5 homologous genes of crambe in the developing seeds: The expression level of Arabidopsis LPAT1–5 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 CaLAPT2. WT = wild type [Colour figure can be viewed at wileyonlinelibrary.com]
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, DraI, 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, DraI, 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 respectively. If the light intensity dropped below 150 W·m⁻², it was compensated with an extra 100 W·m⁻² supplemental light.

#### 2.5.2 qPCR

Two pairs of primers specific to the crambe LPAT2 genes were developed based on the cDNA sequences in C. abyssinica (Gl: 124378834), and two pairs of primers of Arabidopsis LPAT2 gene were developed on the cDNA sequences of AtLPAT2 (Gl:145339616). The gene β-actin 2 (Gl: 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

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis LPAT2</td>
<td>AtLPAT2_F1</td>
<td>GGT GGT TGC AGA AAC CTT GT</td>
</tr>
<tr>
<td>(Gl:145339616)</td>
<td>AtLPAT2_R1</td>
<td>AGC ATG TTC TTT GCC CAT T</td>
</tr>
<tr>
<td></td>
<td>AtLPAT2_F2</td>
<td>GGT GGT TGC AGA AAC CTT GT</td>
</tr>
<tr>
<td></td>
<td>AtLPAT2_R2</td>
<td>GAG CAT GTT TTT TGC CCA TT</td>
</tr>
<tr>
<td>Arabidopsis β-actin 2 (Gl:20465834)</td>
<td>AtActin2_F</td>
<td>GATGGAGACCTCGAAAACCA AAAAAAGCTCTGGGCACTT</td>
</tr>
<tr>
<td></td>
<td>AtActin2_R</td>
<td>GCC ATT GCC TGT AAT GAA GA</td>
</tr>
<tr>
<td>Crambe LPAT2</td>
<td>CalLPAT2_F1</td>
<td>CGC ATT GCC TGT AAT GAA GA</td>
</tr>
<tr>
<td>(Gl:124378834)</td>
<td>CalLPAT2_F2</td>
<td>CCA GTC ATA GCG TGG TCC AT</td>
</tr>
<tr>
<td></td>
<td>CalLPAT2_R1</td>
<td>TTC ATC CTT TGG CCA ATT TC</td>
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<tr>
<td></td>
<td>CalLPAT2_R2</td>
<td>CCA CAA AAA GGG CTA ACC AG</td>
</tr>
<tr>
<td>Crambe FAD2</td>
<td>CaFAD2-F1</td>
<td>CCGTGAAAGCTCTCCAGATAT</td>
</tr>
<tr>
<td></td>
<td>CaFAD2-R1</td>
<td>CGTGGACTATCAGAAGCCGCA</td>
</tr>
</tbody>
</table>
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 μl of hexane to extract the oleic compositions (at room temperature). After centrifuging at 18,000 g for 5 min, 200 μl of the hexane supernatant was taken into the GC vial for triacylglycerol analysis by the high temperature column. Subsequently, 40 μl 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-μl 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, 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 Xhol, 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
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).
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.

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-plot 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.
targeting of the encoding sequence of NHXXXXD box, FP/VEGTR
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The present research also indicated that the LPAT2 expression of
present research showed that, in the developing seeds, the
LPAT2 always has much higher expression levels in various tissues
endoplasmic reticulum. Among the genes encoding LPAT isoforms,
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

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 siliquae) 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 NHXXXXD 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 strat-
egy. 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 + LaLPAT + 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 transform-
lation 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 path-
way engineering alternatively for improving crambe seed-oil quality
and economical value of crambe cultivation.
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DISCLOSURES

There is no conflict of interest regarding the paper.

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**SUPPORTING INFORMATION**

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