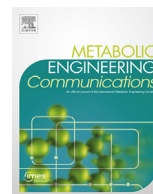




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Co-expressing *Eranthis hyemalis* lysophosphatidic acid acyltransferase 2 and elongase improves two very long chain polyunsaturated fatty acid production in *Brassica carinata*



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ABSTRACT

Docosadienoic acid (DDA, 22:2–13,16) and docosatrienoic acid (DTA, 22:3–13,16,19) are two very long chain polyunsaturated fatty acids (VLCPUFAs) that are recently shown to possess strong anti-inflammatory and anti-tumor properties. An ELO type elongase (EhELO1) from wild plant *Eranthis hyemalis* can synthesize the two fatty acids by sequential elongation of linoleic acid and alpha-linolenic acid, respectively. Seed-specific expression of this gene in oilseed crop *Brassica carinata* produced a considerable amount of DDA and DTA in transgenic seeds. However, these fatty acids were excluded from the *sn*-2 position of triacylglycerols (TAGs). To improve the production level and nutrition value of the VLCPUFAs in the transgenic oilseed crop, a cytoplasmic lysophosphatidic acid acyltransferase (EhLPAAT2) for the incorporation of the two fatty acids into the *sn*-2 position of triacylglycerols was identified from *E. hyemalis*. RT-PCR analysis showed that it was preferentially expressed in developing seeds where *EhELO1* was exclusively expressed in *E. hyemalis*. Seed specific expression of *EhLPAAT2* along with *EhELO1* in *B. carinata* resulted in the effective incorporation of DDA and DTA at the *sn*-2 position of TAGs, thereby increasing the total amount of DDA and DTA in transgenic seeds. To our knowledge, this is the first plant LPAAT that can incorporate VLCPUFAs into TAGs. Improved production of DDA and DTA in the oilseed crop using *EhLPAAT2* and *EhELO1* provides a real commercial opportunity for high value agriculture products for nutraceutical uses.

1. Introduction

Triacylglycerols (TAGs) are storage lipids in seed oil plants consisting of three fatty acids (FAs) esterified to a glycerol backbone that can vary with chain length, number and position of unsaturated bonds as well as functional groups. TAGs with common FAs such as 16:0, 18:0, 18:1–9, 18:2–9,12, and 18:3–9,12,15 in oilseed crops are predominantly used in food industry while TAGs with unusual FAs that differ from those common FAs in terms of chain length, number and position of unsaturated bonds or possession of functional groups are increasingly used in non-food industry (Thelen and Ohlrogge, 2002).

Biosynthesis of TAGs in plants involves the sequential acylations of fatty acids to a glycerol backbone. It starts with the first acylation of an acyl-CoA to the *sn*-1 position of glycerol-3 phosphate (G3P) catalyzed by G3P acyltransferase (GPAT, EC 2.3.1.15), giving lysophosphatidic acid

(LPA), which is followed by the second acylation of an acyl-CoA to the *sn*-2 position catalyzed by lysophosphatidic acid acyltransferase (LPAAT, EC 2.3.1.51), giving phosphatidic acid (PA). PA is then dephosphorylated by PA phosphatase (PAP, EC 3.1.3.4) to diacylglycerol (DAG) that is finally acylated with an acyl-CoA at the *sn*-3 position by DAG acyltransferase (DGAT, EC 2.3.1.20), giving a triacylglycerol (TAG). In addition, TAG can also be synthesized through an acyl-CoA independent pathway where an acyl group from the *sn*-2 position of a phospholipid is transferred to the *sn*-3 position of DAG catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT, EC 2.3.1.43). These acyl-CoA acyltransferases vary with structure and substrate specificity for selective acylation of fatty acids to a glycerol backbone; thus play a decisive role in determining chemical structure, physical properties and potential utility of plant storage oils (Bates et al., 2013).

Lysophosphatidic acid acyltransferase (LPAAT), also known as 1-acyl-*sn*-glycero-3-phosphate acyltransferase (AGPAT) is one of the

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Abbreviations

CoA	coenzyme A
DAG	diacylglycerol
DDA	docosadienoic acid
DTA	docosatrienoic acid
ELO	Fatty acid elongase
FAs	Fatty acids
FAMES	Fatty acids methyl esters
GC	gas chromatography
LPA	lysophosphatidic acid
LPAAT	Lysophosphatidic acid acyltransferase
MAG	monoacylglycerol
PA	phosphatidic acid
TAGs	triacylglycerols
TFA	total fatty acids
TLC	thin layer chromatography
VLCPUFAs	very long chain polyunsaturated fatty acids

acyltransferases specific for positioning a fatty acid at the *sn*-2 position of glycerolipids. In animals including humans, multiple LPAAT homologues exist, each plays unique biochemical and biological functions in specific tissues (Bradley and Duncan, 2018). In plants, LPAATs can have different substrate specificity and compartment localization (Ichihara et al., 1987; Sun et al., 1988; Oo and Huang, 1989; Knutzon et al., 1995; Lassner et al., 1995; Arroyo-Caro et al., 2013). According to the intracellular localization, two types of LPAATs have been identified in plants, plastidic LPAAT and cytoplasmic LPAAT. Most plant species have only one plastidic LPAAT but can have multiple cytoplasmic LPAATs that are associated with cytosolic membrane systems such as endoplasmic reticulum and mitochondrial outer membrane. Plant cytoplasmic LPAAT-As have been cloned from *Limnanthes douglasii*, *Arabidopsis thaliana*, *Brassica napus*, *Echium pitardii*, and *Ricinus communis*. They are normally expressed ubiquitously in plants with broad substrates (Brown et al., 2002a,b; Kim et al., 2005; Maisonneuve et al., 2010; Arroyo-Caro et al., 2013; Mañas-Fernández et al., 2013; Chen et al., 2016) with two exceptions that cytoplasmic LPAAT-As from *R. communis* and *Vernicia fordii* have specific activity towards ricinoleic acid and α -eleostearic acid, respectively (Shockey et al., 2019). Plant cytoplasmic LPAAT-Bs have been identified in *L. douglasii* (Brown et al., 1995; Hanke et al., 1995) and *Cocos nucifera* (Knutzon et al., 1995) where they are usually involved in the incorporation of unusual FAs into storage TAGs in seeds with an exception that a cytoplasmic LPAAT-B from *R. communis* is constitutively expressed and exhibits a broad range of substrates (Arroyo-Caro et al., 2013). In *Arabidopsis* genome, four cytoplasmic LPAAT-As and one plastidic LPAAT, but no cytoplasmic LPAAT-B are annotated. The plastidic LPAAT (AtLPAAT1) is essential for embryo development (Kim and Huang, 2004; Yu et al., 2004). Two cytoplasmic LPAAT-As (AtLPAAT2 and AtLPAAT3) possess LPAAT activity with unknown biological function (Kim et al., 2005). Another two cytoplasmic LPAAT-As (AtLPAAT4 and AtLPAAT5) are ER-associated LPAATs involved in the biosynthesis for phospholipids and TAGs during the nitrogen starvation (Angkawijaya et al., 2019).

Docosadienoic acid (DDA, 22:2–13,16) and docosatrienoic acid (DTA, 22:3–13,16,19) are two new very long chain polyunsaturated fatty acids (VLCPUFAs) that have been recently shown to possess strong anti-inflammatory and antitumor properties (Chen et al., 2020). An ELO-like elongase (EhELO1) has been recently identified from *Eranthis hyemalis* (Meesapyodsuk et al., 2018) that can sequentially elongate linoleic acid and linolenic acid to DDA and DTA, respectively. Expression of the gene in an oilseed crop *Brassica carinata* produced a considerable level of the two VLCPUFAs. However, stereospecific analysis of transgenic seed oil indicated the two VLCPUFAs were completely excluded

from the *sn*-2 position of TAGs, which has limited the production level and nutritional use. To improve the production of the two target fatty acids in transgenic oilseed crops, this study further identified a cDNA (EhLPAAT2) from *E. hyemalis* that encodes a polypeptide with high homology to *Arabidopsis* LPAAT2 and LPAAT3. Expression of this gene in an *E. coli* strain deficient in LPAAT activity showed that it could complement the defective phenotype. Seed-specific expression of this gene in *B. carinata* where EhELO1 was already expressed improved not only the incorporation of DDA and DTA at the *sn*-2 position of TAGs, but also the total amount of the two fatty acids reaching up to 35% of total fatty acids in transgenic seeds. This result has paved the way for a large scaled production of the two healthy VLCPUFAs in oilseed crops for nutraceutical uses.

2. Results

2.1. Fatty acid composition at the *sn*-2 of TAGs from transgenic *B. carinata* seeds expressing an *E. hyemalis* EhELO1

Our previous report showed that seed-specific expression of a single ELO type elongase from *E. hyemalis* in *B. carinata* produced a considerable amount of two new VLCPUFAs, DDA and DTA (Meesapyodsuk et al., 2018). Examination of fatty acid compositions in lipid classes of transgenic seed oil showed that both DDA and DTA were almost exclusively accumulated in TAGs (>99%) with only a trace amount found in other classes (<1%) (Table S2). Further stereospecific distribution analysis of fatty acids in TAGs by lipase digestion revealed that the two target VLCPUFAs were completely excluded from the *sn*-2 position of TAGs in the transgenic seeds, although the precursor fatty acids for the EhELO1 elongation such as 18:2–9,12 and 18:3–9,12,15 were abundantly found at the position in both wild type (70% of total fatty acids) and transgenic *B. carinata* (46% of total fatty acids). On the other hand, DDA at the *sn*-2 position of TAGs from the seeds of *E. hyemalis* was detected at 77% of total fatty acids (Fig. 1 and Table 1). This result clearly indicates that in contrast to the native plant, host plant *B. carinata* lacks the capacity to incorporate the two new fatty acids into the *sn*-2 position of TAGs.

2.2. Identification and sequence analysis of LPAATs from *E. hyemalis*

Absence of DDA and DTA at the *sn*-2 position of TAGs in transgenic *B. carinata* implies that the endogenous LPAATs might not be able to acylate these fatty acids onto the position of glycerolipids. To improve the production in transgenic plants, we thus aimed at identifying LPAATs from native plant *E. hyemalis* that could incorporate the VLCPUFAs onto the *sn*-2 position of TAGs. To do this, RNA-seq approach was first used to establish a transcriptome database from total RNA isolated from developing seeds of *E. hyemalis*. Different types of previously biochemically characterized LPAATs from plant species such as *A. thaliana* (Korbes et al., 2016), *L. douglasii* (Brown et al., 2002a), *C. nucifera* (Knutzon et al., 1995) and *R. communis* (Arroyo-Caro et al., 2013) were then used as queries to search the RNAseq database. Two contigs homologous to query sequences were identified. One contig (EhLPAAT2) combining 146 transcripts was comprised of a full open reading frame and highly homologous to *Arabidopsis* AtLPAAT2, while the other contig (EhLPAAT1) combining 12 transcripts was partial and exhibited a high homology to *Arabidopsis* AtLPAAT1. A method for rapid amplification of cDNA ends (RACE) was thus used to retrieve the 5' missing end of EhLPAAT1. Sequence analysis of the two full-length cDNAs revealed EhLPAAT1 was 1,023 bps coding for a protein of 340 amino acids with a calculated molecular mass of 37.7 kDa, and EhLPAAT2 was 1,161 bps coding for a protein of 386 amino acids with a calculated molecular mass of 43.3 kDa. The two deduced polypeptides shared only 9% of amino acid identity to each other. Homology searching of NCBI protein databases showed that EhLPAAT1 showed very high sequence identity to *Arabidopsis* AtLPAAT1 (79%) and *B. napus* BnLPAAT1 (81%), whereas EhLPAAT2 had very high sequence identity to *Arabidopsis* AtLPAAT2 (79%) and *B. napus*

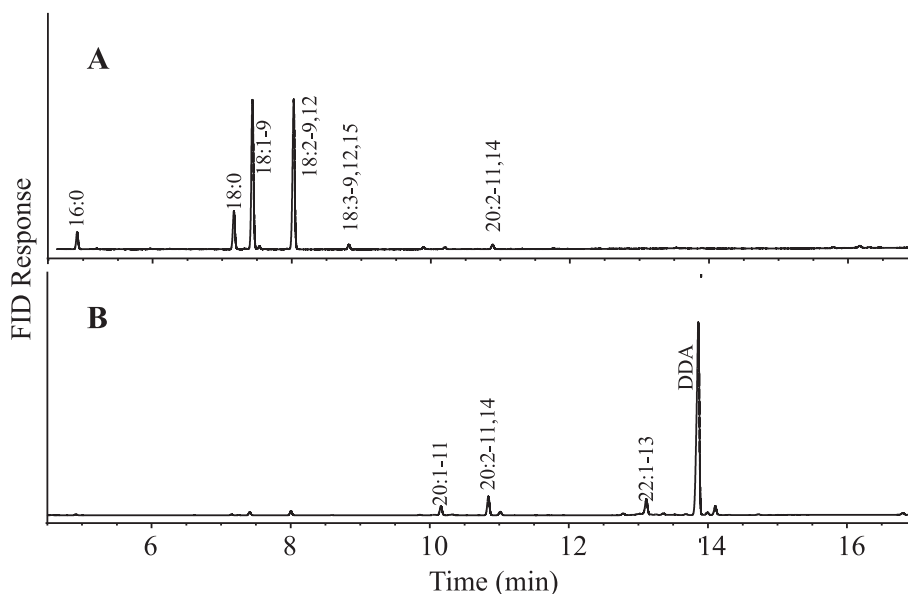


Fig. 1. GC analysis of fatty acids at the sn-2 position of seed TAGs from transgenic *B. carinata* expressing *EhELO1* (A) and from *E. hyemalis* (B).

Table 1

Fatty acyl compositions (mol %) at the sn-2 of TAGs from mature seeds of transgenic *B. carinata* expressing *EhELO1* and of native plant *E. hyemalis*. Each value represents the mean of triplicate \pm standard deviation.

Fatty acid composition	<i>B. carinata</i> WT	Transgenic <i>B. carinata</i> expressing <i>EhELO1</i>	<i>E. hyemalis</i>
C16:0	1.0 \pm 0.19	3.6 \pm 0.70	Trace
C18:0	1.4 \pm 0.23	7.6 \pm 2.12	Trace
C18:1-9+	26.7 \pm 0.52	38.7 \pm 3.08	1.06 \pm 0.06
C18:1-11			
C18:2-9,12	55.5 \pm 0.57	40.0 \pm 3.69	1.51 \pm 0.17
C18:3-9,12,15	15.4 \pm 0.43	6.0 \pm 2.87	ND
C20:0		0.6 \pm 0.24	ND
C20:1-11		0.6 \pm 0.17	3.01 \pm 0.08
C20:2-11,14		2.2 \pm 0.68	6.58 \pm 0.20
C20:3-5,11,14		ND	1.26 \pm 0.06
C20:3-11,14,17		0.3 \pm 0.08	ND
C22:1-13		ND	5.69 \pm 0.16
C22:2-13,16		ND	77.74 \pm 0.52
C24:1		0.7 \pm 0.08	ND

BnALPAAT2 (82%). Sequence alignment of EhLPAAT1 and EhLPAAT2 with related LPAATs from species including *A. thaliana*, *B. napus*, *R. communis*, *S. cerevisiae*, *E. coli*, *Limnanthes douglasii*, *Cocos nucifera*, *Chlamydomonas reinhardtii*, *Synechocystis* sp. PCC 6803, *Synechococcus* sp. WH8102, and *Nostoc* sp. PCC7120 indicated that a phosphate acyltransferase (PlsC) domain with two motifs NHX₄D and EGTX were highly conserved (Fig. S1). The two motifs are believed to be the catalytic site and acyl acceptor binding site, respectively (Heath and Rock, 1998; Lewin et al., 1999). Transmembrane topology analysis revealed that EhLPAAT1 possessed two transmembrane domains (115–137 and 202–224) and EhLPAAT2 had three transmembrane domains (15–37, 306–325, and 335–357), which were similar to those previously reported in AtLPAAT1 and AtLPAAT2, respectively (Korbes et al., 2016). Subcellular localization prediction analysis indicated that EhLPAAT1 was targeted to plastids by a signal polypeptide of 24 amino acids at the N-terminus while EhLPAAT2 was targeted to cytoplasmic endoplasmic reticulum (Sahu et al., 2020).

Phylogenetic analysis of related LPAAT sequences from prokaryotic and eukaryotic organisms showed that homologous LPAATs could be classified into two large groups. The members of the first group included

EhLPAAT1, plant plastidic LPAAT1, plant cytoplasmic LPAAT-B, *E. coli* LPAAT, yeast LPAAT, microalga LPAAT, and cyanobacterial LPAAT. The members of the second group contained EhLPAAT2 and the rest of plant cytoplasmic LPAATs. This result implies that the first group of plant LPAAT1 and LPAAT-B might share a common ancestor with the prokaryotic LPAATs exhibiting the ancient origin, while the second group of plant cytoplasmic LPAATs might emerge thereafter, being divergent from the first group (Korbes et al., 2016) (Fig. 2 and Table S3).

2.3. Complementation assay of EhLPAAT2 in an *E. coli* LPAAT mutant

As EhLPAAT2 encodes an endoplasmic reticulum LPAAT, next we aimed at the analysis of functional properties of this gene. To determine if EhLPAAT2 functioned as an active LPAAT enzyme, an *E. coli* mutant strain SM2-1 (*plsC^{ts}*) was exploited for complementation assays (Coleman, 1990). *E. coli* has a single *PlsC* gene encoding an LPAAT that is essential for membrane glycerolipid biosynthesis, and the knockout of this gene is lethal (Coleman, 1990). Therefore, functional complementation assays of EhLPAAT2 were carried out in a SM2-1, a temperature sensitive mutant able to grow at the permissive temperature of 30 °C, but not at the non-permissive temperature 42 °C, which has widely been used to examine the function of plant LPAAT enzymes, including plastidial and extraplasmidial LPAATs (Hanke et al., 1995; Brown et al., 2002a; Kim and Huang, 2004; Kim et al., 2005; Arroyo-Caro et al., 2013). Complementation test of EhLPAAT2 showed that the mutant transformed with the empty vector could grow only at 30 °C, but not at 42 °C. However, the mutant cell expressing EhLPAAT2 was able to grow at both 30 °C and 42 °C (Fig. 3). This result indicates that EhLPAAT2 from *E. hyemalis* could complement the defective phenotype of *plsC^{ts}* and restore the LPAAT function for the biosynthesis of membrane lipids in *E. coli*.

2.4. EhLPAAT2 was preferentially expressed in developing seeds of *E. hyemalis*

To determine spatial expression of EhLPAAT2 in *E. hyemalis*, total RNAs were isolated from leaves, stems, seed pods, and developing seeds, and then used in semi-quantitative RT-PCR analysis. *EhELO1* encoding a polyunsaturated fatty acid elongase in the biosynthesis of DDA was exclusively expressed in developing seeds. *EhLPAAT2* was preferentially, but not exclusively expressed in developing seeds. It also exhibited some expression in stems and seed pods. This coordinating expression pattern of *EhELO1* and *EhLPAAT2* in developing seeds implies EhLPAAT2

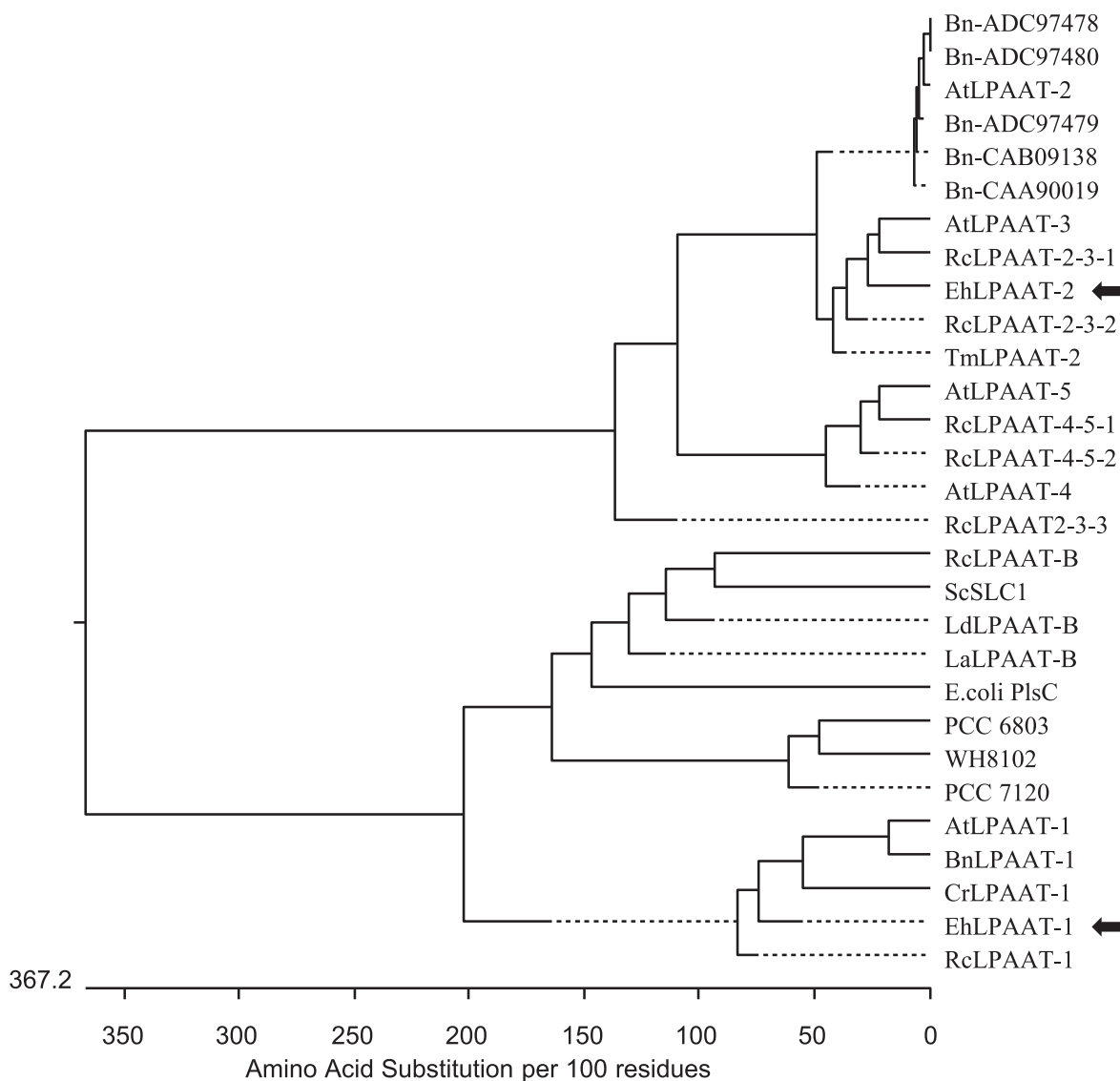


Fig. 2. Phylogenetic analysis of EhlPAATs and related sequences. The species and GenBank accession numbers of the sequences used are indicated in Table S3.

possesses acylating activity towards VLCPUFAs, which would warrant the efficient assembly of the VLCPUFAs after they are synthesized in a temporospatial manner (Fig. 4).

2.5. Expression of EhlPAAT2 enhanced production of DDA and DTA in transgenic *B. carinata*

To investigate the capacity of EhlPAAT2 in the assembly of VLCPUFAs into TAGs, thereby enhancing the production of DDA and DTA in transgenic plants, the full-length cDNA was expressed under the control of a seed specific promoter in *B. carinata* where the EhELO1 in the biosynthesis of DDA and DTA was previously expressed in the same seed-specific manner. Fourteen transgenic plants were produced, and their authenticities were confirmed by the presence of both transgenes by genomic DNA PCR analysis. Fatty acid analysis of transgenic seeds showed that thirteen out of the fourteen transgenic lines outperformed the production of the two target VLCPUFAs, increasing the amount in a range of 7% to 33% over the control (Fig. 5a and b). Detailed analysis of fatty acid compositions in five elite lines showed that adding EhlPAAT2 not only improved the production of DDA and DTA, but also produced more C24 VLCPUFAs such as 24:2–15,18 and 24:3–15,18,21 (Table 2, Fig. S2). *B. carinata* expressing EhELO1 alone accumulated about 28% of

DDA and DTA while *B. carinata* co-expressing EhELO1 and EhlPAAT2 produced an average of 35% with a range from 33% to 38% of DDA and DTA, representing an increase from 17% to 33% of two target fatty acids. If two C24 VLCPUFAs (24:2–15,18 and 24:3–15,18,21) were taken into calculation, the increase would be in a range of 20% to 44%.

2.6. EhlPAAT2 incorporated DDA and DTA into the sn-2 position of TAGs in transgenic *B. carinata*

To investigate if the enhanced amount of VLCPUFAs was actually attributed to EhlPAAT2 for acylating these fatty acids onto the sn-2 position of TAGs, TAGs were isolated from transgenic seeds and subjected to the digestion by a TAG lipase. Fatty acid analysis of the MAG fraction from the digestion showed that EhlPAAT2 effectively incorporate both DDA and DTA into the sn-2 position of TAGs in transgenic seeds. As compared with *B. carinata* expressing EhELO1 alone where DDA and DTA were completely excluded from the sn-2 position of TAGs, *B. carinata* co-expressing EhELO1 and EhlPAAT2 could incorporate both DDA and DTA into the sn-2 position of TAGs (Fig. 6). DDA and DTA together accounted for the average of 4.2% of the total fatty acids at the position (Table 3). This result clearly indicates that EhlPAAT2 from *E. hyemalis* could acylate DDA and DTA into the sn-2 position of the glycerolipids in

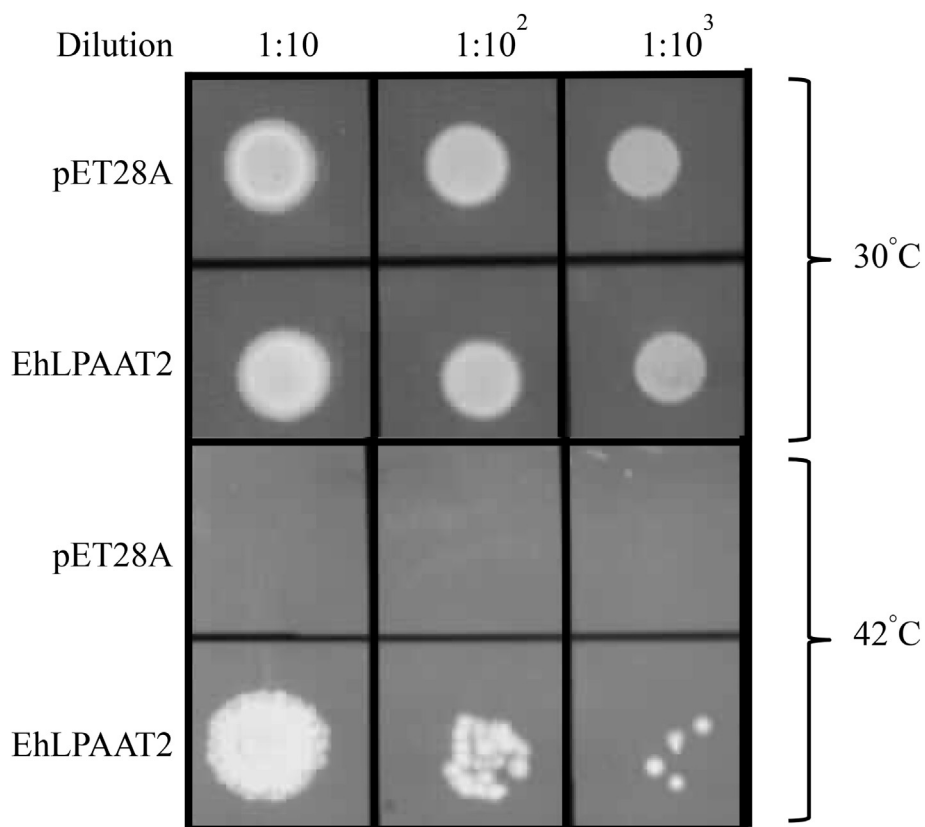


Fig. 3. Complementation of the *E. coli* LPAAT mutant (SM2-1) by EhLPAAT2 from *E. hyemalis*. SM2-1 cells were transformed with EhLPAAT2/pET28A (EhLPAAT2) or with empty vector (pET28A). Five microliters of each cells were spotted onto plates containing 0.1 mM IPTG with a series of dilutions. Plates were incubated at 30 °C or 42 °C.

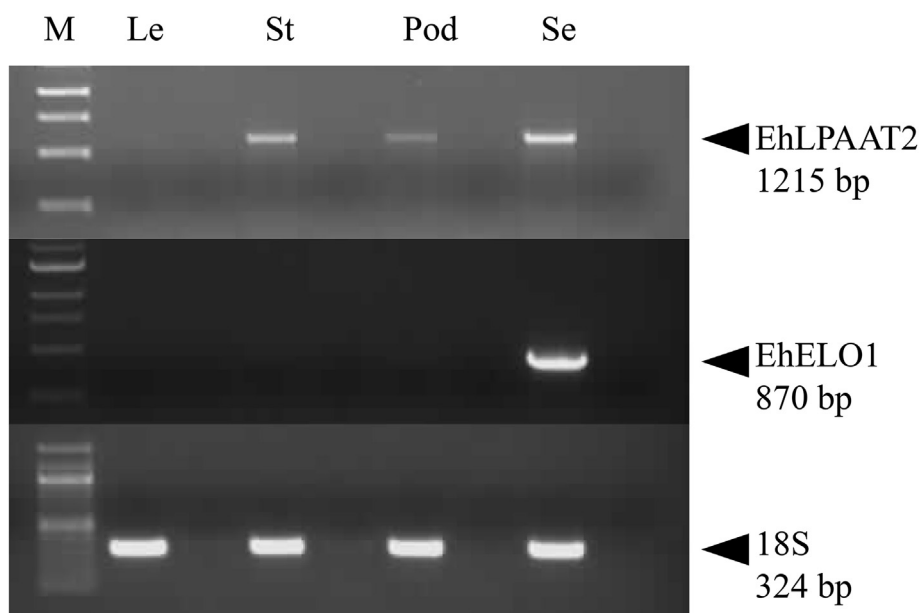


Fig. 4. Semi-quantitative RT-PCR analysis of the transcripts from various tissues, Le (leaves), St (stems), Pod (seed pods) and Se (developing seeds) with primers specific for EhLPAAT2, EhELO1 and 18S RNA. M: size markers.

transgenic *B. carinata*. Furthermore, EhLPAAT2 could also improve the incorporation of C20 VLCPUFAs (20:2-11,14 and 20:3-11,14,17) into the *sn*-2 position of TAGs. The average amounts of 20:2-11,14, and

20:3-11,14,17 were 4.7% and 1.4% in EhLPAAT transgenics, representing an increase of 114% and 367%, respectively, relative to those in the control. The collective amount of four VLCPUFAs including

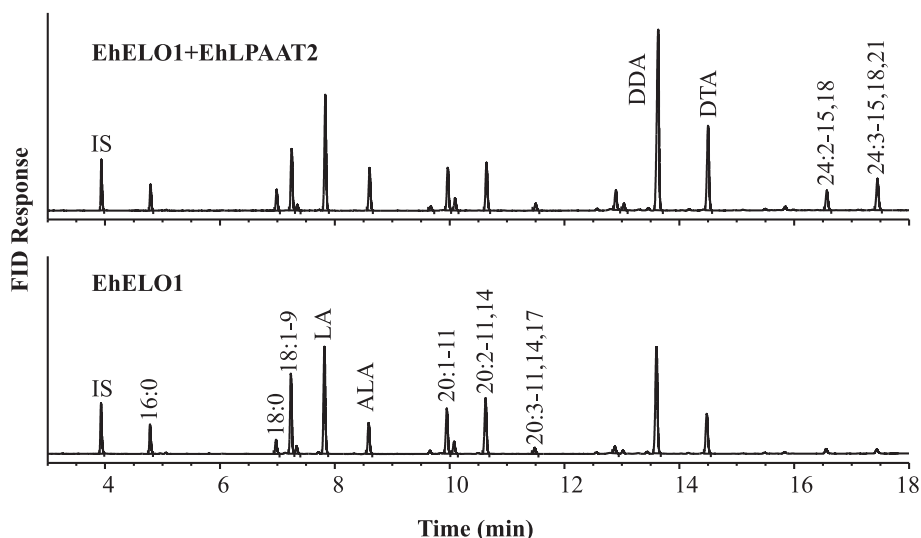


Fig. 5a. GC analysis of total fatty acids in transgenic seeds expressing EhELO1 alone and co-expressing EhELO1 and EhLPAAT2. IS, internal standard 15:0.

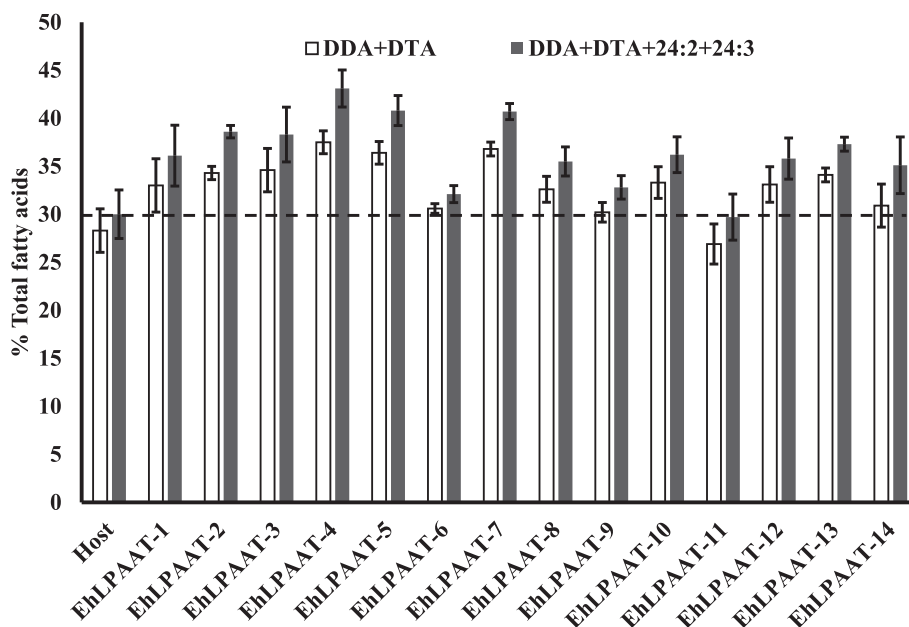


Fig. 5b. The amount of C22 and C24 VLCPUFAs in transgenic *B. carinata* co-expressing EhELO1 and EhLPAAT2.

20:2–11,14, 20:3–11,14,17, DDA, and DTA at the *sn*-2 position of TAGs were increased more than threefold in the transgenics. These results unambiguously show that EhLPAAT2 from *E. hyemalis* could effectively incorporate both C20 and C22 VLCPUFAs into the *sn*-2 position of TAGs (Table 4).

2.7. Expression of EhLPAAT2 also resulted in increasing seed weight, and seed oil in transgenic *B. carinata*

To evaluate if the enhanced production of VLCPUFAs by EhLPAAT2 would affect other phenotypes, the five EhLPAAT2 transgenic lines and the control line were simultaneously grown under the same conditions. No distinguishable morphological and developmental changes were observed between the control and these EhLPAAT2 transgenic plants. However, when seed weight and seed oil were compared, significant changes could be observed between them. In *B. carinata* expressing EhELO1 alone, the average weight per seed was 4.10 mg while the amount was increased to 5.23 mg in EhLPAAT2/EhELO1 transgenic

lines. In *B. carinata* expressing EhELO1 alone, the average oil content per seed was at 1.20 mg while in *B. carinata* co-expressing EhELO1 and EhLPAAT2, the average of oil per seed in the transgenic lines was at 1.56 mg with a range of 1.28 mg and 1.92 mg, representing an average increase of 30% relative to the control. However, no significant difference in the relative percentage of oil content was observed between the control and EhLPAAT2 transgenic lines due to the concurrent increase of seed weight and seed oil (see Table 4).

3. Discussion

Although DDA and DTA were recently shown to possess strong anti-inflammatory, anti-proliferating, and anti-cancer properties (Chen et al., 2020), there is no natural rich source of these fatty acids for commercial use. The first plant ELO-like elongase identified from *E. hyemalis* can synthesize DDA and DTA by sequential elongations of linoleic acid and alpha-linolenic acid. Seed-specific expression of this gene in oilseed crop *B. carinata* produced a considerable amount of the

Table 2

Fatty acid composition (mol %) of mature seeds of five transgenic *B. carinata* lines co-expressing EhELO1 and *EhLPAAT2*. Each value represents the mean of five seeds \pm standard error.

Fatty Acid	Host	T1 -1	T1-2	T1-3	T1-4	T1-5	Means
16:0	3.6 \pm 0.13	3.8 \pm 0.41	3.6 \pm 0.29	3.1 \pm 0.12	2.8 \pm 0.22	2.6 \pm 0.06	3.2 \pm 0.23
18:0	2.7 \pm 0.08	3.2 \pm 0.32	2.8 \pm 0.03	2.6 \pm 0.19	2.4 \pm 0.17	2.3 \pm 0.07	2.7 \pm 0.16
18:1-9	15.7 \pm 1.80	9.7 \pm 2.58	8.8 \pm 0.32	10.4 \pm 1.93	10.1 \pm 1.42	11.3 \pm 0.85	10.1 \pm 0.41
18:1-11	1.1 \pm 0.12	1.3 \pm 0.10	1.1 \pm 0.17	1.1 \pm 0.09	0.8 \pm 0.07	0.7 \pm 0.02	1.0 \pm 0.11
18:2	15.9 \pm 0.88	18.5 \pm 0.59	15.7 \pm 0.64	15.5 \pm 0.80	13.3 \pm 0.59	13.0 \pm 0.25	15.2 \pm 0.99
18:3	5.0 \pm 0.07	4.8 \pm 0.73	6.3 \pm 0.09	5.4 \pm 0.20	5.8 \pm 0.34	5.3 \pm 0.10	5.5 \pm 0.25
20:0	0.7 \pm 0.02	0.6 \pm 0.03	0.7 \pm 0.04	0.6 \pm 0.03	0.5 \pm 0.13	0.5 \pm 0.01	0.6 \pm 0.04
20:1-11	11.2 \pm 0.80	6.9 \pm 1.67	7.9 \pm 0.82	8.3 \pm 1.73	7.9 \pm 1.07	9.8 \pm 0.85	8.2 \pm 0.47
20:1-13	1.9 \pm 0.13	1.9 \pm 0.13	1.7 \pm 0.08	1.8 \pm 0.16	1.5 \pm 0.10	1.4 \pm 0.04	1.7 \pm 0.09
20:2	8.9 \pm 0.45	9 \pm 0.50	7.2 \pm 0.32	8.0 \pm 0.31	7.2 \pm 0.40	7.4 \pm 0.24	7.8 \pm 0.34
20:3	1.0 \pm 0.09	1.1 \pm 0.13	1.2 \pm 0.02	1.2 \pm 0.12	1.3 \pm 0.13	1.4 \pm 0.05	1.2 \pm 0.05
22:0	0.0 \pm 0.00	0.2 \pm 0.01	0.3 \pm 0.01	0.2 \pm 0.00	0.0 \pm 0.00	0.0 \pm 0.00	0.1 \pm 0.06
22:1-13	1.2 \pm 0.65	1.8 \pm 0.25	2.8 \pm 0.41	2.3 \pm 0.15	2.4 \pm 0.27	2.8 \pm 0.03	2.4 \pm 0.19
22:1-15	0.4 \pm 0.21	0.9 \pm 0.12	0.8 \pm 0.02	0.8 \pm 0.08	0.7 \pm 0.05	0.7 \pm 0.04	0.8 \pm 0.04
22:2(DDA)	20.6 \pm 1.90	25.1 \pm 1.61	23.1 \pm 0.33	24.3 \pm 2.30	24.2 \pm 1.25	24.2 \pm 0.96	24.2 \pm 0.32
22:3(DTA)	7.7 \pm 0.54	7.9 \pm 1.39	11.2 \pm 0.55	10.3 \pm 0.11	13.2 \pm 0.74	12.2 \pm 0.25	11.0 \pm 0.91
24:0	0.0 \pm 0.00	0.0 \pm 0.00	0.2 \pm 0.03	0.1 \pm 0.01	0.0 \pm 0.00	0.0 \pm 0.00	0.1 \pm 0.04
24:1-15	0.0 \pm 0.00	0.2 \pm 0.02	0.4 \pm 0.01	0.3 \pm 0.01	0.1 \pm 0.03	0.0 \pm 0.00	0.2 \pm 0.07
24:2	0.8 \pm 0.13	1.4 \pm 0.17	1.5 \pm 0.15	1.4 \pm 0.25	2.0 \pm 0.42	1.6 \pm 0.14	1.6 \pm 0.11
24:3	0.9 \pm 0.14	1.7 \pm 0.30	2.7 \pm 0.03	2.3 \pm 0.37	3.6 \pm 0.66	2.8 \pm 0.29	2.6 \pm 0.31

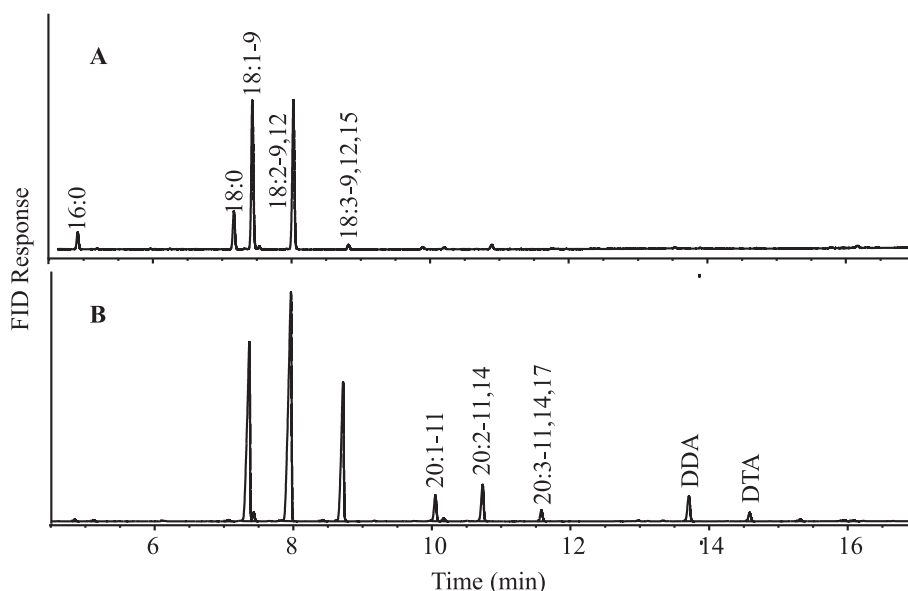


Fig. 6. GC analysis of fatty acids at the sn-2 position of TAGs from transgenic *B. carinata* seeds expressing EhELO1 alone (A) and co-expressing EhELO1 and EhLPAAT2 (B).

two VLCPUFAs (Meesapyodsuk et al., 2018). However, stereospecific distribution analysis showed the two fatty acids were completely excluded from the sn-2 position of TAGs. To improve the production of the two fatty acids, we resorted to the same native plant for LPAATs that might be able to incorporate the fatty acids into the position of TAGs. Using an RNA-seq technology, we identified two LPAATs transcripts *EhLPAAT1* and *EhLPAAT2* in the developing seeds of *E. hyemalis*. *EhLPAAT1* codes for plastidic LPAAT with a plastid-targeting sequence of 24 amino acids at the N-terminus that might be involved in the biosynthesis of prokaryotic glycerolipids such as monogalactosyl diacylglycerol, digalactosyldiacylglycerol in chloroplast, while *EhLPAAT2* codes for endoplasmic reticulum LPAAT that is probably involved in the biosynthesis of eukaryotic glycerolipids including triacylglycerols. Complementation test of *EhLPAAT2* in *E. coli* showed it could complement the phenotype of a temperature sensitive mutant defective in LPAAT and restore its function for the biosynthesis of membrane glycerolipids. Expression of *EhLPAAT2* along with EhELO1 in *B. carinata* showed that it significantly improved the production of target VLCPUFAs in transgenic

seeds. Adding this gene to the host plant where EhELO1 was expressed for the biosynthesis of DDA and DTA could increase the production of the two VLCPUFAs from 28% to 35% of the total fatty acids on average. DDA and DTA at the sn-2 position of TAGs was increased from almost zero in the control to the average of 4.1% in the transgenics. Interestingly, this LPAAT is active toward not only DDA and DTA, but also 18:3-9,12,15, 20:2-11,14, and 20:3-11,14,17. To our knowledge, this is the first plant functionally characterized LPAAT that can effectively incorporate VLCPUFAs at the sn-2 position of TAGs.

Triacylglycerol is a vital energy and carbon skeleton reserve in oil-seeds for early plant growth and development, also an important bio-resource for food and industrial uses. Stereospecific distribution of fatty acids in TAGs determines the functionality and utility of this bioresource. Biosynthesis of TAGs involves the sequential acylations of fatty acids onto a glycerol backbone catalyzed by a variety of acyltransferases. LPAAT is one of them that can specifically acylate a fatty acid onto the sn-2 position of a backbone converting 1-acyl-sn-glycerol-3-phosphate to PA, an intermediate in the synthesis of glycerolipids (J Browse and

Somerville, 1991). Therefore, substrate specificity of this enzyme has been recognized to be a limiting factor in the incorporation of specialty fatty acids into the position of glycerolipids, thereby constraining the production of TAGs with unusual fatty acids. For instance, in some plant species, specific LPAATs are required to incorporate erucic acid (Brown et al., 1995), lauric acid (Knutzon et al., 1995), ricinoleic acid (Chen et al., 2016) and sterculic acid (Yu et al., 2014) into triacylglycerols. In this study, co-expression of EhELO1 and EhLPAAT2 could effectively synthesize and incorporate two unusual VLCPUFAs, DDA and DTA into the *sn*-2 position of TAGs, thereby improving the production of the two fatty acids in *B. carinata* seeds. It is noteworthy that LPAAT is not the only enzyme that can incorporate a fatty acid into the *sn*-2 position of TAGs. Lysophosphatidyl choline acyltransferase (LPCAT) (Chen et al., 2007) is another acyltransferase that can also incorporate a fatty acid into the *sn*-2 position of TAGs through a PC-derived DAG pathway. In the pathway, LPCAT acylates a fatty acid to the *sn*-2 position of 1-acyl-sn-glycerol-3-phosphocholine giving PC, from which derived DAG is then used to synthesize TAG catalyzed by DGAT. However, it remains to be investigated if this pathway is also employed in incorporating the VLCPUFAs at the *sn*-2 position of TAGs in plants.

Metabolic engineering of TAGs in oilseed crops with specialty fatty acids for nutritional and industrial applications has recently attracted much attention. One of popular strategies is to use acyl-CoA acyltransferases in the Kennedy pathway for “pulling” fatty acids into storage TAGs. For instance, overexpression of DGAT1 and LPAAT has been widely used to increase oil content in oilseed plants (Zou et al., 1997; Taylor et al., 2002; Maisonneuve et al., 2010; Shockey et al., 2019) where fatty acid profiles are very much determined by substrate specificity of these acyl-CoA acyltransferases (Mavraganis et al., 2010). In this study, co-expression of EhLPAAT2 and EhELO1 resulted in the significant increase of not only two new VLCPUFAs, but also seed weight and seed oil relative to those in the control with EhELO1 alone. The increased seed weight and oil content in transgenics might be simply due to the enhanced ability in “pulling” fatty acids including VLCPUFAs into TAGs by this novel LPAAT. This result reaffirms the effectiveness of utilizing co-evolved enzymes in the biosynthesis and assembly of unusual fatty acids for the production of specialty oil in transgenic oilseed crops.

4. Materials and methods

4.1. Materials

Standard fatty acids and lipids were purchased from Nu-Chek-Prep, Inc. (Elysian, MN, USA). Q5 DNA polymerase, restriction enzymes and deoxynucleoside triphosphate (dNTP) were obtained from New England Biolabs (Ipswich, MA, USA). DNA-modifying enzymes and TRIzol reagent were from Thermo Fisher Scientific (Waltham, MA, USA). HP Taq DNA polymerase, nucleic acid purification and DNA extraction kits were obtained from Bio Basic Inc. (Markham, ON, Canada). RNA isolation kit,

Table 3

Fatty acyl compositions (mol %) at the *sn*-2 position of TAGs in mature seeds of five transgenic lines co-expressing EhELO1 and EhLPAAT2. Means is the average of five lines (T1-1 to T1-5 ± standard deviation).

Fatty acid composition	Host	T1-1	T1-2	T1-3	T1-4	T1-5	Means
C16:0	3.6 ± 0.70	0.4	0.4	0.6	0.2	0.6	0.4 ± 0.17
C18:0	7.6 ± 2.12	0.4	0.3	0.6	0.2	0.6	0.4 ± 0.18
C18:1-9+ C18:1-11	38.7 ± 3.08	34.4	29.4	25.7	27.9	28.3	29.1 ± 3.23
C18:2-9,12	40.0 ± 3.69	43.7	40.6	43.6	38.6	40.5	41.4 ± 2.20
C18:3-9,12,15	6.0 ± 2.87	9.7	17.8	15.2	19.5	13.4	15.1 ± 3.83
C20:0	0.6 ± 0.24	0	0	0	0	0	0 ± 0.00
C20:1-11	0.6 ± 0.17	2.5	3.3	3.5	3.2	4.0	3.3 ± 0.54
C20:2-11,14	2.2 ± 0.68	4.6	4.0	5.2	4.5	5.1	4.7 ± 0.49
C20:3-11,14,17	0.3 ± 0.08	0.6	1.0	1.3	1.3	2.6	1.4 ± 0.75
C22:1-13	0 ± 0.00	0	0	0	0	0	0 ± 0.00
C22:2-13,16	0 ± 0.00	3.3	2.5	3.6	3.4	4.0	3.4 ± 0.55
C22:3-13,16,19	0 ± 0.00	0.4	0.7	0.7	1.1	0.9	0.8 ± 0.26
C24:1	0.7 ± 0.08	0	0	0	0.1	0	0 ± 0.00

RNase-Free DNase set and DNeasy plant mini kit were purchased from Qiagen (Toronto, ON, Canada). Smarter RACE cDNA amplification kit and intermediated vector, pGEM-T were purchased from Clontech (Mountain View, CA, USA) and Promega (Madison, WI, USA), respectively. Oligo primers were synthesized from Sigma-Aldrich (Oakville, ON, Canada). HPLC and GC grade solvents and *E. coli* media were either obtained from VWR (Edmonton, AB, Canada) or Fisher Scientific (Ottawa, ON, Canada). All other chemicals and plant media were purchased from Sigma-Aldrich Ltd (Oakville, ON, Canada).

4.2. Cloning of full length cDNA coding for lysophosphatidic acid acyltransferase from *E. hyemalis*

Plant LPAATs from class A and class B sequences were used as queries to search the RNA-seq library prepared from *E. hyemalis* developing seeds. Five class A LPAATs are from *A. thaliana*, AtLPAAT1 (NP194787), AtLPAAT2 (NP567052), AtLPAAT3 (NP175537), AtLPAAT4 (NP565098) and AtLPAAT5 (NP188515) (Kim and Huang, 2004; Yu et al., 2004) and three class B LPAATs are from *L. douglasii* (Q42870) (Brown et al., 1995; Hanke et al., 1995), *C. nucifera* (Q42670) (Knutzon et al., 1995), and *R. communis* (NP001310689) (Arroyo-Caro et al., 2013). Two LPAAT-like cDNAs (one full length *EhLPAAT2* and one partial *EhLPAAT1*) were identified from the database. The 5' cDNA ends of *EhLPAAT1* was obtained using the Smarter RACE cDNA amplification kit following the manufacturer's instruction with primer DM972 (Table S1). The resulting sequence information from RNA-seq data of *EhLPAAT1* and *EhLPAAT2* along with 5' RACE result of *EhLPAAT1* were used to design the specific primers including start and stop codon with appropriate restriction sites (Table S1) for retrieving the full-length cDNAs by reverse transcriptase PCR using total RNA extracted from *E. hyemalis* developing seeds (Meesapyodsuk and Qiu, 2014). Two full-length genes were cloned into a pGEM-T intermediate vector, and named *EhLPAAT1/pGEM-T*, and

Table 4

The amount of oil and mass per seed in transgenics co-expressing EhLPAAT2. Values were reported as the means of three biological replicates with standard error. The means with the same letters are not statistically significantly different, while the means with different letters are significantly different according to the statistical analysis ($P < 0.05$).

Line	The amount of oil (µg/seed)	Seed weight (mg/seed)	Oil content (µg/mg)
Control	1202.8 ± 45.17 ^a	4.10 ^a	293.4 ± 11.02 ^a
T1-1	1600.4 ± 96.76 ^b	5.0	320.1 ± 19.35 ^a
T1-2	1919.7 ± 106.53 ^b	6.66	288.2 ± 16.00 ^a
T1-3	1372.0 ± 41.01 ^b	4.42	310.4 ± 9.28 ^a
T1-4	1627.4 ± 87.01 ^b	5.27	308.8 ± 16.51 ^a
T1-5	1284.4 ± 108.18 ^a	4.80	267.6 ± 22.54 ^a
T1- mean	1560.8 ± 62.56 ^b	5.23 ^b	298.5 ± 8.28 ^a

EhLPAAT2/pGEM-T, respectively.

4.3. Expression analysis of *EhELO1* and *EhLPAAT2* by RT-PCR

For relative expression analysis, total RNAs from leaves, stems, and seed pods were isolated from *E. hyemalis* plants using the RNeasy Plant Mini Kit. Total RNA from developing seeds used for both expression study and cDNA cloning of *EhLPAAT1* and *EhLPAAT2* was extracted by TRIzol reagent and then purified on the RNeasy Plant Mini column. Genomic DNA contamination of RNA samples was removed by on-column DNaseI digestion using RNase free DNase. To investigate gene expression by semi-quantitative RT-PCR, first-strand cDNA was synthesized from 1 µg of total RNA using SuperScriptIII (Tan et al., 2011).

4.4. Complementation assay of *EhLPAAT1* and *EhLPAAT2* in the *E. coli* *plsC* mutant (SM2-1)

E. coli SM2-1, a temperature-sensitive mutant of *plsC* gene that encodes an LPAAT, was used to test the activity of *E. hyemalis* LPAATs. To express *EhLPAAT2* gene in SM2-1 strain, the coding region was released with *EcoRI* digestion from intermediate plasmid, EhLPAAT2/pGEM-T, and then cloned into an *E. coli* expression vector pET28A under the control of a T7 promoter. The orientation of recombinant plasmid, EhLPAAT2/pET28A, was confirmed by digestion and sequencing. The recombinant plasmid and vector control were individually transformed into *E. coli* mutant, SM2-1. Mutant transformants were first grown at 30 °C overnight. The overnight cultures were diluted to the same starting concentration at OD₆₀₀ of 0.5. The serial dilutions made from the initial concentration were then spotted onto two selective agar plates with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). One was incubated at 30 °C and the other was incubated at 42 °C (Xie et al., 2018).

4.5. Expression of *EhLPAAT2* in *B. carinata*

Mature seeds of *Eranthis hyemalis* were purchased from Vesey's Seeds Ltd, York, PE, Canada. A elite transgenic line of *Brassica carinata* (pEP-T3) expressing *EhELO1* along with a herbicide phosphinothricin (PPT) resistance gene (Meesapyodsuk et al., 2018) was used as a host to express *EhLPAAT2*. The full length *EhLPAAT2* was released from EhLPAAT2/pGEM-T by *EcoRI* and cloned into a plant expression vector under the control of a seed specific conlinin promoter with NptII selectable marker (Subedi et al., 2015). The construct was delivered into pEP-T3 by an *Agrobacterium* infiltration method using cotyledon as explants as described previously (Cheng et al., 2010). The new transgenic plants containing EhLPAAT2 were selected on kanamycin and PPT plates. Transgenic plants were grown in the growth cabinet at 22 °C under a 16-h-light (120 µE m⁻² s⁻¹)/8-h-dark photoperiod.

4.6. Seed oil content and fatty acid analysis of plant seeds

To analyze total fatty acids compositions of mature seeds, each seed was crushed with glass rod and directly transmethylated to FAMES by 2 ml of 1% H₂SO₄ in methanol (v/v) at 80 °C for 2 h in the presence of 50 µg of triptadecanoin as internal lipid standard. The samples were cooled down on ice and then added with 1 ml of 0.9% NaCl and 2 ml of hexane for the extraction of FAMES. The FAMES were analyzed on an Agilent 6890N gas chromatography equipped with a DB-23 column (30 m × 0.25 mm) with 0.25-µm film thickness (J&W Scientific). The column temperature was maintained at 160 °C for 1 min, and then raised to 240 °C at a rate of 4 °C/min. The lipid amount per seed was calculated based on the amount of internal standard (Meesapyodsuk et al., 2015).

4.7. Analysis of the fatty acid composition of the sn-2 position of triacylglycerols from plant seeds

About 100 mg of plant mature seeds in 1 ml of isopropanol with

0.01% butylated hydroxytoluene were incubated at 75 °C for 15 min to inactivate phospholipase. After cooling down on ice, seed samples were ground with glass rod. Total lipids were extracted by a mixture of solvents with chloroform, methanol and water (1:1:0.8, v/v/v). The fatty acid composition of the sn-2 position of TAG was determined by lipase digestion. About 3 mg of oil in a glass tube was dissolved in 250 µl of diethyl-ether and 1 ml of a buffer containing 0.1 M Tris-HCl pH 7.7, and 5 mM CaCl₂. Reactions were initiated by adding 15 µl of *Rhizomucor miehei* lipase. The mixture was incubated at 37 °C for 90 min in a thermomixer with 400 rpm. The reaction was stopped by adding 100 µl of 0.15 M acetic acid and the digestion was extracted by adding 3.5 ml of chloroform:methanol (2:1, v/v). After mixing and centrifuging at 2000 rpm for 5 min, the organic phase was recovered into a new glass tube, dried under N₂ and resuspended in either chloroform or hexane. MAG was separated from other lipids on Silica gel G60 plates using a solvent system of hexane:diethylether:acetic acid (40:60:1). Bands of the MAG fraction were scrapped from TLC plate and directly transesterified according to the method described above. The fatty acid compositions were analyzed by GC (Meesapyodsuk et al., 2015).

Accession numbers

Genbank accession numbers for cDNAs of *EhLPAAT1* and *EhLPAAT2* are MW755447 and MW755448, respectively.

Author contributions

DM, XQ, and RGC designed the experiments. DM, YC and SY performed the experiments. DM and XQ analyzed the data and wrote the paper. All authors approved the final manuscript.

Declaration of competing interest

THE authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mec.2021.e00171>.

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