Research Paper

Glycerol-3-phosphate acyltransferase 4 is essential for the normal development of reproductive organs and the embryo in Brassica napus

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Abstract

The enzyme sn-glycerol-3-phosphate acyltransferase 4 (GPAT4) is involved in the biosynthesis of plant lipid polyesters. The present study further characterizes the enzymatic activities of three endoplasmic reticulum-bound GPAT4 isoforms of Brassica napus and examines their roles in the development of reproductive organs and the embryo. All three BnGPAT4 isoforms exhibited sn-2 acyltransferase and phosphatase activities with dicarboxylic acid-CoA as acyl donor. When non-substituted acyl-CoA was used as acyl donor, the rate of acylation was considerably lower and phosphatase activity was not manifested. RNA interference (RNAi)-mediated down-regulation of all GPAT4 homologues in B. napus under the control of the napin promoter caused abnormal development of several reproductive organs and reduced seed set. Microscopic examination and reciprocal crosses revealed that both pollen grains and developing embryo sacs of the B. napus gpat4 lines were affected. The gpat4 mature embryos showed decreased cutin content and altered monomer composition. The defective embryo development further affected the oil body morphology, oil content, and fatty acid composition in gpat4 seeds. These results suggest that GPAT4 has a critical role in the development of reproductive organs and the seed of B. napus.

Key words: Brassica napus, cutin biosynthesis, embryo development, female fertility, GPAT, reproductive organ.

Introduction

The sn-glycerol-3-phosphate acyltransferase (GPAT) family is involved in acyl-lipid biosynthesis and plays a pivotal role in plant development (Chen et al., 2011a). GPAT catalyses the transfer of a fatty acid moiety from acyl-coenzyme A (acyl-CoA) or acyl-acyl carrier protein (acyl-ACP) to the sn-1 or 2 position of sn-glycerol 3-phosphate (G3P) forming lysophosphatidic acid (LPA). Additionally, certain GPATs also possess phosphatase activity and thus result in the formation of monoacylglycerols (MAGs) (Yang et al., 2012). Based on subcellular localization, GPATs can be categorized into three types: (i) the plastidial soluble GPATs; (ii) the mitochondrial membrane-bound GPATs; and (iii) the endoplasmic reticulum (ER) membrane-bound GPATs. The plastidial GPAT (also annotated as ATS1) was first cloned from squash (Ishizaki et al., 1988). Its homologues have also been cloned and characterized from several plant species including pea (Pisum sativum) and Arabidopsis (Weber et al., 1991; Nishida et al., 1993; Xu et al., 2006). Plastidial GPATs were shown...
to catalyse only the \( sn-1 \) acylation of G3P and are primarily involved in the prokaryotic glycerolipid biosynthesis pathway. Three isoforms encoding the mitochondrial membrane-bound GPATs (i.e. GPATs 1–3) have been cloned in *Arabidopsis* (Zheng et al., 2003). Among them, AtGPAT1 was essential for tapetal differentiation and male fertility, and *Arabidopsis gpat1* T-DNA mutants had altered fatty acid compositions in the storage and membrane lipids of flower buds, pollen grains, and seeds (Zheng et al., 2003). Recently, AtGPAT1 was shown to possess only \( sn-2 \) acyltransferase activity with acyl substrate preference for mono behenoyl (22:0)-CoA and mono 22:0 \( \alpha,\omega \)-dicarboxylic acid (DCA)-CoA (Yang et al., 2012).

There are six members of the ER-bound GPATs in *Arabidopsis* (i.e. AtGPAT4–AtGPAT9 (Zheng et al., 2003; Li et al., 2007; Gidda et al., 2009)). AtGPAT4, 5, 6, and 8 were found to be involved in the synthesis of extracellular lipid polymers (i.e. cutin and suberin) by providing acylglycerol found to be involved in the synthesis of extracellular lipid polymers (i.e. cutin and suberin) by providing acylglycerol esters (Beisson et al., 2007; Li et al., 2007; Li-Beisson et al., 2009). In *in vitro* enzyme assays, AtGPAT4, 6, and 8 were shown to be bi-functional for acyltransferase (mainly at the \( sn-2 \) position) and phosphatase activities resulting in \( sn-2 \)-MAG formation. In comparison, AtGPAT5 and 7 only exhibited strong preference for \( sn-2 \) acylation without phosphatase activity (Yang et al., 2012). Although the physiological and catalytic functions of AtGPAT9 are still unclear, it was predicted to be involved in membrane and storage lipid biosynthesis (Gidda et al., 2009).

Interestingly, GPAT6 was shown to play a role in the development of the tapetum ER profile and pollen grains (Li et al., 2012), suggesting that in addition to extracellular lipid polymer biosynthesis, the ER-bound GPATs may have other unknown physiological functions.

As a close relative of *Arabidopsis*, *Brassica napus* is an important oilseed crop with a complex polyploid genetic background. Previously, three homologues of *GPAT4* were cloned in *B. napus* and their important roles in cutin accumulation were revealed, via an RNA interference (RNAi) approach to down-regulate *GPAT4* expression constitutively in vegetative tissues [under the direction of the *Cauliflower mosaic virus* (CaMV) 35S promoter] (Chen et al., 2011b). The present study further investigates the enzymatic properties of the three GPAT4 isoforms and the functions of the isoforms in the development of reproductive organs and embryos of *B. napus*. The expression profiles of *B. napus* genes encoding several mitochondrial and ER-bound GPATs were investigated using quantitative real-time PCR (qRT-PCR). *GPAT4* exhibited the highest expression level among all investigated *GPAT* genes in maturing embryos. All three BnGPAT4 isoforms showed \( sn-2 \) acyltransferase and phosphatase activities when DCA-CoA was used as acyl donor. Acylation rates, however, were considerably lower with non-substituted acyl-CoA but, under these conditions, phosphatase activity was not manifested. With an RNAi silencing approach, under the direction of a napin promoter, abnormal development of the inflorescence, reduced seed set, decreased embryonic cutin and seed oil content, and an altered fatty acid composition were observed in the *B. napus gpat4* lines. In addition, examination of pollen and the embryo sac, and reciprocal crosses, suggested that down-regulation of *GPAT4* caused impaired female fertility, which is the main reason for reduced seed set. The results strongly suggest that in addition to cutin synthesis, GPAT4 plays important physiological roles in reproductive organ and embryo development in *B. napus*.

### Materials and methods

#### Plant growth

*Brassica napus* double haploid line (DH12075) plants were grown in a growth chamber at 23 °C under an 18 h day/6 h night cycle.

#### Synthesis of 16:0 DCA-CoA and 16:0 DCA-MAGs

The substrate 16:0 DCA-CoA was synthesized according to Kawaguchi et al. (1981) using 16:0 DCA, carbonyldiimidazole, and tetrahydrofuran. Briefly, 5 μmol of 16:0 DCA and 6 μmol of carbonyldiimidazole were mixed in 0.2 ml of tetrahydrofuran. The mixture was sealed, and rapidly stirred at 1000 rpm under room temperature for 30 min. After the solvent had been evaporated under N2, the residue was dissolved in 0.2 ml of tetrahydrofuran/H2O (2:1, v/v) and 5 μmol of freshly prepared CoASH was added, with the final pH being adjusted to 7.0–7.5. The product 16:0 DCA-CoA was precipitated by 7% perchloric acid, and the remaining 16:0-DCA was extracted by hexane. The 16:0 DCA-CoA precipitate was washed with 0.7% perchloric acid and dissolved in 0.01 M NaOAc/EtOH (1:1, v/v; pH 5.2), filled with N2, and stored at −20 °C (Yang et al., 2010). The purity of the 16:0 DCA-CoA was checked by thin-layer chromatography (TLC) with butanol:water:acetic acid (5:3.2, v/v/v) as the developing solvent and the concentration was determined by gas chromatography/mass spectrometry (GC/MS) with internal standard (17:0 methyl ester) after methylation with 2% sulphuric acid in methanol.

The \( sn-1 \) and \( sn-2 \) 16:0 DCA-MAGs were synthesized according to Yang et al. (2010). For the synthesis of \( sn-2 \) 16:0 DCA-MAG, a mixture containing 1 ml of tetrahydrofuran, 9 μmol of 4-(dimethylamino) pyridine, 18 μmol of N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride, 27 μmol of 1,3-benzodioxane, and 18 μmol of 16:0-DCA was stirred at room temperature for 24 h. Subsequently, the reaction mixture was diluted with 5 ml of ethyl acetate and washed with 3 ml of water and 3 ml of saturated NaCl solution. The organic layer was extracted and dried under N2. To remove the benzodioxane group, 1–4 ml of trimethyl borate and 0.0015 g of boric acid powder were added to the mixture, which was stirred at 98 °C for 20 min, dried under N2, and heated at 98 °C for another 10 min. The product was extracted with 5 ml of diethyl ether and washed three times with 2 ml of water. The organic extract was applied to boric acid-TLC in a developing solvent of 2-butanone:acetone (1:1, v/v) and detected using UV light to locate the MAG spots. The 16:0-DCA-MAG was eluted with 100 ml of diethyl ether and washed three times with 2 ml of water. The organic extract was applied to the TLC plate and developed in 1-methyl-1,3-dioxolane-4-methanol was used to replace 1,3-benzodioxane.

#### Heterologous expression of BnGPAT4s in the yeast gat1Δ strain and enzyme assays

BnGPAT4s were produced in yeast strain gat1Δ for *in vitro* enzyme assay (Chen et al., 2011b). The GPAT enzyme assay was performed according to the method reported in Yang et al. (2010). A 20 μg aliquot of yeast microsomal protein was incubated with 0.5 mM \([1^{14}C(U)]G3P\) (0.1 μCi) and 45 μM acyl-CoA in a buffer containing 37.5 mM Tris-HCl (pH 7.5), 2 mM MgCl2, 4 mM NaF, 1 mM dithiothreitol (DTT), and 0.1% bovine serum albumin (BSA; w/v) at room temperature for 10 min. The reactions were quenched with 5 μl of
Aniline blue staining of pollen tube growth in *B. napus* plants

Mature seeds were enclosed in a piece of wet filter paper at 4 °C overnight. The cotyledons were cut into 1–2×1–2 mm pieces, which were fixed in 0.1 M sodium cacodylate buffer pH 7.2, with 2% paraformaldehyde and 3% glutaraldehyde for 24 h. The samples were washed with 0.1 M sodium cacodylate buffer to remove fixative and post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 3 h. The samples were washed three times with 0.1 M sodium cacodylate buffer after the post-fixing and dehydrated in a graded ethanol series (50, 70, 90, and 100% ethanol). The samples were then treated with a graded ethanol/proplylene oxide series (2:1, 1:1, and 1:2, v/v), infiltrated into a propylene oxide/Spurr resin mixture (1:1, v/v) overnight and embedded in 100% Spurr resin for 24 h. The samples were sectioned using an ultramicrotome. Sections of ~200 nm thickness were placed onto glass slides and observed under light microscopy with a Leica DMRXA microscope coupled with a Nikon DXM1200 camera. Sections of 70–90 nm thickness were placed onto 300 mesh copper grids, and stained with 4% uranyl acetate in 50% ethanol for 30 min and then with Reynolds’s lead citrate solution for 8 min. The treated sections were observed under a Morgagni 268 (Philips-FEI) transmission electron microscope (TEM) at 80 kV accelerating voltage.

**Seed oil extraction and analysis**

Seed samples were boiled in 1 ml of isopropanol for 10 min at 80 °C. After cooling to room temperature, the samples were ground with a homogenizer in 1 ml of hexane and 2 ml of 3:2 hexane/isopropanol (HIP, v/v), containing 1 mg ml⁻¹ tri-17:0-triacylglycerol (TAG) as a standard. After centrifugation at 1500 g, the upper hexane phase of the mixture was extracted, and 2 ml of 7:2 (v/v) HIP were added to the remaining mixture for a second extraction. The hexane extracts were combined, evaporated under nitrogen, and then a portion of the resulting extract was incubated with 1 ml of methanolic HCl for 1 h at 80 °C to prepare the fatty acid methyl esters.

Fatty acid methyl esters were extracted twice with 1 ml of hexane and resuspended in iso-octane for GC/MS analysis, which was performed with an Agilent 6890N gas chromatograph with an Agilent 5975 Inert Mass Selective Detector. Chromatographic separation was achieved using a capillary DB-23 column (30 m×0.25 mm×0.25 μm) with a constant helium flow rate of 1.2 ml min⁻¹ and with temperature programmed from 90 °C to 180 °C at 10 °C min⁻¹. The inlet was operated in splitless mode at 290 °C. For mass spectra detection, the solvent delay was 4 min and ionization energy was 70 eV; a scan mode range of 30–350 amu was used for data acquisition.

**Results**

**Expression patterns of the GPAT family members of *B. napus***

To obtain sequence information of the *B. napus* GPAT genes, the cDNA sequences encoding eight confirmed *Arabidopsis* membrane-bound GPAT isoforms, including three isoforms located in the mitochondria (AtGPAT1–AtGPAT3) (Zheng et al., 2003) and five isoforms located in the ER (AtGPAT4–AtGPAT8) (Zheng et al., 2003; Li et al., 2007), were used to search the *B. napus* expressed sequence tag (EST) database (Megablast, NCB! (Zhang et al., 2000) for the corresponding orthologues. Note that the putative GPA9 was not included in the present study because a catalytic function for this category of GPAT has not been established.

As an allotetraploid closely related to *Arabidopsis*, *B. napus* has been shown to have on average six copies of the *Arabidopsis* conserved genome segments with a number of exceptions of less or more than six copies, due to genome-wide rearrangement after polyploidy (Parkin et al., 2005). In the present study, the EST sequence analysis...
also revealed that each *Arabidopsis* GPAT gene had multiple orthologues (sequence identity >90%) in the *B. napus* genome, with the exception of *AtGPAT7* and *AtGPAT8*. In the case of *AtGPAT7*, no *B. napus* EST sequences with a significant identity were identified. For *AtGPAT8*, which has a cDNA sequence identity of >80% to *AtGPAT4*, the corresponding *B. napus* orthologues are identical to those found for *AtGPAT4* (Chen et al., 2011b). Thus, six clusters of ESTs were identified and designated BnGPAT1, 2, 3, 4, 5, and 6, which are orthologous to *AtGPAT1*–*AtGPAT6*, respectively.

SYBR-green qRT-PCR was used to investigate the transcript abundance of the BnGPAT genes in different tissues and organs of *B. napus*. To simplify the qRT-PCR experiment and to have a robust comparison of the expression patterns between different BnGPAT genes, a pair of primers was designed based on the highly conserved regions of individual BnGPAT EST clusters, such that the overall transcript abundance of each BnGPAT orthologous cluster (i.e. BnGPAT1–BnGPAT6) could be obtained. As shown in Fig. 1, the expression levels of BnGPAT1–BnGPAT6 were investigated not only in maturing embryos, but also in several other plant tissues/organs, including seedlings, leaves, dehiscent anthers, and flowers. In maturing embryos, BnGPAT1 and BnGPAT4 appeared to be more transcriptionally active than the other BnGPAT genes, suggesting a potentially important role for these isoforms during embryo development. In contrast, BnGPAT2 and BnGPAT3 exhibited very low transcript abundance in all investigated samples. BnGPAT5 appeared to be expressed specifically in the anthers at a high level. BnGPAT6 was expressed exclusively in vegetative tissues and flower organs.

The gene expression profiles suggested that BnGPAT1 and BnGPAT4 were the most active genes within the GPAT family during seed development. A previous study showed that GPAT1 deficiency in *Arabidopsis* caused reduced seed set and altered fatty acid composition in the seed oil (Zheng et al., 2003). In the following study, the focus was on the enzymatic and physiological characterization of the three BnGPAT4 isoforms to gain insight into their roles in reproductive organ and seed development.

### Three BnGPAT4 isoforms exhibited sn-2 acyltransferase and phosphatase activities

In *Arabidopsis*, GPAT4 possesses both sn-1 and sn-2 acyltransferase and phosphatase activities (Yang et al., 2010). To investigate the catalytic functions of BnGPAT4s, *in vitro* enzyme assays were performed with individual recombinant BnGPAT4 isoforms. The expression levels of individual BnGPAT4 isoforms were first checked by western blotting to confirm that all three isoforms accumulated at a similar level per milligram of yeast crude microsomal protein (Supplementary Fig. S1 at JXB online). Crude microsomal fractions of yeast expressing individual BnGPAT4 isoforms were assayed with [14C(U)]G3P and palmitoyl (16:0)-CoA or 16:0 DCA-CoA. As shown in Fig. 2A, when the three BnGPAT4 isoforms were assayed with 16:0-CoA as acyl donor, the product was only LPA, with BnGPAT4-A1 exhibiting the highest preference for 16:0-CoA. In contrast, when 16:0 DCA-CoA was used as the acyl donor, both LPA and MAG were formed, with BnGPAT4-C1 exhibiting the highest preference for 16:0 DCA-CoA (Fig. 2B). The DCA-CoA-fuelled reactions, however, proceeded at substantially higher rates than for reactions using 16:0-CoA. In addition, acylation with DCA-CoA was required to activate the phosphatase activity of the GPAT4 isoforms. Further study on the regiospecificity of the DCA-MAGs indicated that the majority of MAG formed during the enzyme assay was sn-2 DCA-MAG (Fig. 2C).
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Down-regulation of GPAT4 in B. napus

A previous study has shown that all three BnGPAT4 homologues (>93% identical in open reading frame (ORF) sequences) were expressed during the embryo maturation process, although with different transcript abundance (Chen et al., 2011b). Given that the BnGPAT4 isoforms possess similar catalytic functions, down-regulation of a single BnGPAT4 homologue may not give a detectable phenotype due to the high probability of functional overlap. Thus, to understand the physiological functions of BnGPAT4 genes with a robust approach, an RNAi construct was designed to down-regulate all three BnGPAT4 genes in an embryo-specific fashion. In total, 11 T1 transgenic lines (confirmed by genomic DNA PCR) were generated, and five T2 transgenic lines were studied in detail. SYBR-green qRT-PCR was used to analyse the overall transcript abundance of BnGPAT4 genes and several BnGPAT genes (that encode the ER-bound isoforms) in the RNAi lines using a mixture of developing embryos of the T2 transgenic lines at 40 days after flowering (DAF). As shown in Fig. 3, the expression level of BnGPAT4 homologues was down-regulated by ~65%. Other BnGPAT genes, including BnGPAT6 that has the highest sequence identity (~60%) to BnGPAT4 within the GPAT family, did not change much in expression. This result confirmed that the RNAi construct was specific only to BnGPAT4 homologues.

Brassica napus gpat4 lines exhibited abnormal inflorescence and reduced seed set

Inflorescence development was affected in gpat4 RNAi transgenic lines. In all gpat4 RNAi T1 and T2 transgenic lines, the development of floral buds was partially aborted, particularly on the lower portion of the inflorescence (Fig. 4A, B). Additionally, the development of axillary inflorescence primordia was also severely affected (Fig. 4C, D). Consequently, gpat4 RNAi lines had fewer flowers in comparison with the wild-type plants. Notably, BnGPAT4 was shown to be highly expressed in the inflorescence primordia in a previous study (Chen et al., 2011b). These lines of evidence strongly suggest that BnGPAT4 is pivotal for inflorescence development in B. napus. Additionally, the stigma and style of gpat4 lines also appeared to be larger than those of the wild type (Fig. 4E–G).

The gpat4 RNAi lines also exhibited reduced seed yield. In the T1 generation, a few independent gpat4 RNAi lines were completely sterile. The majority of gpat4 RNAi lines had short and stocky siliques with <10 seeds within each siliqua (Fig. 5B, E, H, I), while the wild-type siliques were much longer, containing on average >20 seeds per siliqua (Fig. 5A, F, G). The B. napus gpat4 seeds also appeared to be larger (Fig. 5J, K) and had significantly increased weight (P<0.001) compared with wild-type seeds (Table 1). In Arabidopsis gpat4 gpat8 double T-DNA lines, reduced seed set was also observed, but it was not as severe as in the B. napus gpat4 lines (Fig. 5C, D).

Down-regulation of GPAT4 affected pollen development in B. napus

To investigate the cause(s) of the reduced seed yield of the B. napus gpat4 lines, the mature pollen grains of the gpat4 lines were first examined under light microscopy and TEM.
As shown in Fig. 6B, under light microscopy, some of the gpat4 pollen grains were deformed or collapsed. Further examination under TEM revealed that in addition to the deformed pollen grains, some of the normal-shaped gpat4 pollen grains had defective exine deposition on the pollen wall (Fig. 6C–F). Considering that B. napus flowers produce an excessive amount of pollen grains to ensure sufficient fertilization, it is possible that the remaining gpat4 pollen grains of normal appearance could enable normal pollination. Thus, the in vivo pollen tube growth of the gpat4 lines was examined. Both wild-type and gpat4 pistils were hand pollinated with their own pollen grains, and then stained with aniline blue at 2 DAF. As shown in Fig. 6G, H, in both wild-type and gpat4 lines, the germinated pollen tubes could reach the ovary locules. Thus, it appeared that although a portion of pollen grains were adversely affected, the remaining viable pollen grains of the gpat4 lines had the ability to germinate in vivo.

Impaired female fertility reduced seed yield in B. napus gpat4 lines

To understand further the reason for the reduced seed yield of the B. napus gpat4 lines, reciprocal crosses were performed between the gpat4 and wild-type B. napus lines. As shown in Fig. 7A, B and Supplementary Table S1 at JXB online, seed development and seed number per silique were normal when wild-type stigmas were pollinated with gpat4 pollen grains. On the other hand, pollination of gpat4 flowers with wild-type pollen grains resulted in fewer seeds per silique, as in the self-pollinated gpat4 plants. These results suggested that the deficiency of GPAT4 in B. napus severely affected female fertility, and the remaining viable gpat4 pollen grains were capable of normal fertilization.

The developing embryo sac of the gpat4 lines was examined further before and after pollination. Unpollinated and pollinated pistils were collected on the first and fourth day of anther dehiscence, respectively. Although it was difficult to distinguish the individual nuclei, the gpat4 embryo sacs exhibited no apparent morphological difference compared with the wild type before pollination (Fig. 7C, D). At 4 DAF, however, most of the gpat4 embryo sacs exhibited arrested development (Fig. 7F) or deformed shapes (Fig. 7G, H). These results indicated that the aborted seed development of gpat4 may be caused by defective embryo sac development after pollination.

Brassica napus gpat4 mature embryos exhibited alterations in cutin content and monomer profile

Previous studies have shown that GPAT4 is involved in biosynthesis of the cuticle layer covering the aerial tissues of the
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Plant (Li et al., 2007). Given that the embryo is also covered by a thin layer of cuticle (Molina et al., 2006), it was of interest to investigate if the cutin content of the gpat4 mutant embryos was affected. As shown in Fig. 8, the total cutin content of the mature embryos of RNAi lines was significantly decreased by >30% compared with the wild-type line. The cutin monomer profile was also changed in the RNAi lines. Among the monomers, 18:1 DCA, 18:2 DCA, and 18:3 9,10,18 hydroxy fatty acid (18:3 9,10,18-OH FA) exhibited significant reductions.

Brassica napus gpat4 seeds exhibited decreased oil content and altered fatty acid composition

The oil content and fatty acid composition of the T2 generation gpat4 seeds were also different from those of the wild type. As shown in Table 1, the seed oil contents (wt%) were significantly decreased (by 12.4–24.1% on a relative basis, \( P<0.05 \)) in the gpat4 lines compared with the wild type and segregant lines. Furthermore, the fatty acid profiles were also different in the gpat4 lines. The molar proportions of oleic acid (18:1 cis \( \Delta 9 \)) were decreased by 4.3–16.5% on a relative basis and the molar proportions of linoleic acid (18:2 cis \( \Delta 9,12 \)) and \( \alpha \)-linolenic acid (18:3 cis \( \Delta 9,12,15 \)) were increased to varying degrees (Table 1, Fig. 9). Similar changes in fatty acid composition were also observed in the T1 seeds (Supplementary Fig. S2 at JXB online).

To investigate the physiological effect of GPAT4 down-regulation on oil body accumulation, cotyledon sections of mature embryos of gpat4 and the wild type were examined under light microscopy and TEM. In wild-type mature embryo cells, the oil bodies were present in the...
Fig. 5. Aborted development of seeds in gpat4 RNAi lines. (A) Comparison between a wild-type plant and a gpat4 line during silique development. A reduced number of siliques was observed in the gpat4 lines. (B) Close-up view of the developing siliques of a gpat4 line. (C, D) Comparison of the developing siliques from Arabidopsis wild-type and gpat4 gpat8 lines. Black arrows in (C) indicate the aborted siliques. Scale bar in (D), 1 mm. (E) Seeds inside a developing gpat4 silique. Scale bar=1 mm. (F–I) Before and after opening of the developing siliques of the wild type (F, G) and gpat4 lines (H, I). Scale bars in (F, G), 1 cm. Scale bars in (H, I), 0.5 cm. (J, K) Comparison of the 10 days after flowering (DAF) J and 25 DAF K developing seeds of the wild type (left) and gpat4 lines (right). Scale bar in (J), 1 mm. Scale bar in (K), 1 cm.

Table 1. Seed oil analysis of the wild-type and gpat4 lines

| Weight per 10 seeds (mg) | Oil content wt% | Fatty acid composition (mol%) | n |
|--------------------------|-----------------|-----------------------------|---|
|                          |                 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 |
| Average                  |                 | 4.626 | 1.960 | 61.360 | 17.707 | 10.830 | 0.405 | 0.667 | 3 |
| SE                       |                 | 0.512 | 1.3 | 0.446 | 0.281 | 0.238 | 0.047 | 0.025 | 4 |
| Average                  |                 | 4.830 | 1.918 | 61.225 | 17.392 | 11.102 | 0.507 | 0.780 | 4 |
| SE                       |                 | 0.330 | 0.9 | 0.234 | 0.074 | 0.138 | 0.027 | 0.007 | 3 |
| Average                  |                 | 5.133 | 1.441 | 51.240 | 24.148 | 14.512 | 0.330 | 0.847 | 4 |
| SE                       |                 | 1.421 | 0.1 | 0.618 | 0.488 | 0.117 | 0.051 | 0.020 | 4 |
| Average                  |                 | 5.299 | 1.308 | 51.497 | 22.774 | 15.365 | 0.356 | 0.835 | 4 |
| SE                       |                 | 1.477 | 0.9 | 0.783 | 0.375 | 0.289 | 0.029 | 0.016 | 3 |
| Average                  |                 | 5.057 | 1.318 | 51.986 | 22.124 | 15.842 | 0.358 | 0.859 | 4 |
| SE                       |                 | 0.908 | 1.1 | 0.909 | 0.583 | 0.355 | 0.031 | 0.011 | 4 |
| Average                  |                 | 4.909 | 1.521 | 57.679 | 20.555 | 11.899 | 0.391 | 0.837 | 4 |
| SE                       |                 | 1.765 | 1.4 | 0.899 | 0.723 | 0.248 | 0.021 | 0.021 | 4 |
| Average                  |                 | 4.650 | 1.269 | 58.744 | 19.479 | 12.306 | 0.327 | 0.932 | 4 |
| SE                       |                 | 0.681 | 1.1 | 0.844 | 0.334 | 0.630 | 0.025 | 0.034 | 4 |
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Periphery of the cells and between the centrally located protein bodies. As shown in Fig. 10A–D, the protein bodies appear as dark blue spheres, surrounded by the oil bodies (light blue or light grey colour) within each cell. There appeared to be more protein bodies present in the gpat4 embryo cells (Fig. 10C, D) than in the wild type (Fig. 10A, B). The ultrastructure of the oil bodies was also different between the wild and gpat4 lines as observed under TEM. The oil bodies in the wild-type embryo cells were pressed against each other into irregular shapes (Fig. 10E–G). In contrast, oil bodies within the gpat4 embryo cells were rounder, smaller, and often disconnected from each other (Fig. 10H–J).

**Discussion**

A previous study of gpat4 RNAi lines under the control of the CaMV35S promoter found that the only detectable phenotype was a cuticle defect on the epidermis (Chen et al., 2011b); however, two (out of 10) T1 lines exhibited abnormal inflorescence development and severely reduced seed yield, the same abnormality observed in the current napin promoter-directed gpat4 RNAi lines. It appeared that the napin promoter-directed RNAi construct was more effective in down-regulating the expression of GPAT4 in the reproductive organs of B. napus. The napin promoter used in the present study originates from a napA gene (a member of the napin gene family, GenBank...
accession no. J02798), which encodes a 1.7S seed storage protein in *B. napus* (Josefsson et al., 1987). The expression patterns of the *napin* gene family or the corresponding promoters have mainly been investigated within the developing seeds (Blundy et al., 1991; Kridl et al., 1991; Ellerstrom et al., 1996); thus, it is not clear whether *napin* genes could also be expressed in other plant organs. Notably, the corresponding orthologous genes in *Arabidopsis* (AT4G27170, AT4G27140, AT4G27160, AT4G27150, and AT5G54740), which encode seed storage albumin proteins, are expressed not only in
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Developing seed but also in pollen grain (Supplementary Fig. S3 at JXB online, via the AtGenExpress Visualization Tool; Schmid et al., 2005). Beside the promoter, another factor that can affect the organ targeting of RNAi is that gene silencing regulated by RNAi can spread locally and systemically (Klahre et al., 2002; Himber et al., 2003; Dong et al., 2011). Thus, it is possible that the RNAi construct was expressed at a higher level than the natural expression level driven by a napin promoter in the reproductive organs, such as the flower primordium and pollen grains. It was previously shown that the BnGPAT4 homologues are expressed at the highest level in the flower primordium among all the investigated vegetative organs (Chen et al., 2011b). Therefore, it is believed that BnGPAT4s are important for the development of flower primordium, possibly with a role in providing the polyester surface layer for this developing organ given the enzymatic activity of the corresponding enzymes. The disruption of normal flower primordium development will subsequently affect the flower organ development. As a result, a series of developmental defect were observed in the flower organs. The B. napus gpat4 lines not only produced collapsed pollen grains but also had pollen grains with reduced deposition of exine. The development of pollen is a complex process, which is known to be closely connected to lipid metabolism within the tapetum and pollen grain itself (Zheng et al., 2003; Wang et al., 2008; Beaudoin et al., 2009). Two previous studies

Fig. 8. Down-regulation of GPAT4 homologues resulted in decreased cutin monomer load. (A) The gpat4 lines had decreased cutin content by >30% (P<0.05) in the mature embryos compared with the wild-type (WT) lines; n=3. (B) The cutin monomer profile of the gpat4 and WT lines; n=3. The error bar denotes the SD. (This figure is available in colour at JXB online.)
in *Arabidopsis* revealed that down-regulation of two other GPAT family members, *AtGPAT1* and *AtGPAT6*, caused defective ER profiles in the tapetum, which further affected nutrient secretion from the tapetum to pollen grains (Zheng *et al.*, 2003; Li *et al.*, 2012). It is possible that *BnGPAT4* is similarly involved in tapetum development so as to affect pollen development.

The *gpat4* seeds not only were larger during development, but also had increased weight at maturity compared with wild-type seeds. This was possibly caused by an increased source-to-sink ratio in the RNAi lines, given that the *B. napus gpat4* lines had much less seed set compared with the wild type due to defective inflorescence development and reduced female fertility of the *gpat4* lines. Although the seed weight increased, the seed oil content (wt%) in the *gpat4* lines was decreased. These results indicate that suppression of *GPAT4* expression may have resulted in restricted use of cellular carbon for storage lipid biosynthesis. Furthermore, in contrast to the wild type, the oil bodies in the *gpat4* mature seeds were rounder, smaller, and disconnected from each other. Previous studies have suggested that oleosin, which is the major protein present in the phospholipid monolayer of oil bodies,
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plays an important role in controlling the size of oil bodies (Ross et al., 1993; Tzen et al., 1993; Ting et al., 1996; Siloto et al., 2006). These studies indicated that higher TAG to oleosin ratios resulted in larger oil bodies, while lower TAG to oleosin ratios resulted in smaller oil bodies. Thus, the smaller and rounder oil bodies in the gpat4 seeds could be caused by a lower TAG to oleosin ratio imparted by the decreased oil content in the seeds.

In the present study, it was demonstrated that the BnGPAT4 isoforms are similar to AtGPAT4 in being able to use DCA-CoA as acyl substrate, and all possess sn-2 acyltransferase and phosphatase activities. Such enzymatic activities were suggested to be related to the role of GPAT4 in cutin biosynthesis in providing intermediates for cutin polymer assembly (Yang et al., 2010, 2012). Cutin, a major component of the cuticle layer, has been found on the epidermis of aerial organs and developing embryos (Molina et al., 2006). As previous studies have only revealed the role of GPAT4 in cuticle formation of vegetative tissues (Li et al., 2007; Chen et al., 2011b), in the present study, it was further demonstrated that down-regulation of BnGPAT4 homologues also affected embryo cutin biosynthesis. It is not clear whether such a substantial reduction in cutin load could potentially affect embryo development. It has been reported that in an Arabidopsis cuticular

Fig. 10. Mature embryo sections of the wild type and gpat4. (A, B) Sections of wild-type embryo cotyledons. (C, D) Sections of gpat4 embryo cotyledons. More protein bodies were present in the gpat4 cotyledon cells than in the wild-type cotyledon cells. (E–G) Transmission electron microscopy (TEM) images of the wild-type cotyledons. The T2 wild-type segregant line exhibited the same cellular morphology. (H–J) TEM images of the gpat4 cotyledons. PB, protein body; OB, oil body. The oil bodies in wild-type cotyledon cells were compacted and connected to each other; in contrast, the oil bodies in gpat4 cotyledon cells were disconnected from each other and appeared to be smaller and rounder than those observed in the wild type.
wax mutant line resurrection1 (rst1), 70% of the seeds underwent arrested embryo development. Interestingly, the surviving shrunked rst1 seeds exhibited a similar phenotype to the gpat4 seeds, which was characterized by reduced seed oil content and altered fatty acid composition (Chen et al., 2005).

Although GPAT can catalyse the non-substituted acyl-CoA-dependent production of LPA, which is the initial step in bringing the glycerol backbone into the TAG synthesis network, there are few experimental data, so far, to demonstrate that the currently known ER GPATs are directly involved in the biosynthesis of storage lipids in plants. On the other hand, the strong sn-2 acyltransferease activity using ω-oxidized acyl-CoAs as substrates of GPAT4 would provide substrates for extracellular lipid polyester biosynthesis rather than feed into the Kennedy pathway (Weselake et al., 2009). However, GPAT4 is not involved in the Kennedy pathway (Marroquin et al., 2014).

In summary, the results from the present study have revealed critical physiological roles for BnGPAT4s in reproductive organ and embryo development. This information adds further to knowledge on the physiological roles of these multifunctional enzymes in plant development.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Western blot of individual BnGPAT4 isoforms expressed in yeast strain gat1Δ.

Figure S2. Fatty acid composition analysis indicated decreased content of 18:1 and increased content of 18:2 and 18:3 in the B. napus gpat4 T1 seed oil.

Figure S3. The expression patterns of Arabidopsis genes encoding seed storage albumin proteins.

Table S1. Seed set analysis of the reciprocal crosses between gpat4 and wild-type B. napus lines.

Table S2. Sequences of primers and the RNAi construct used in the present study.

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