The Brassica Napus Fatty Acid Exporter FAX1-1 Contributes to Biological Yield, Seed Oil Production, and Oil Quality

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Abstract

Background: In the oilseed crop *Brassica napus* (rapeseed), various metabolic processes influence seed oil production, oil quality, and biological yield. However, the role of plastid membrane proteins in these traits has not been explored.

Results: Our genome-wide association study (GWAS) of 520 *B. napus* accessions identified the chloroplast membrane protein-localized FATTY ACID EXPORTER 1-1 (FAX1-1) as a candidate associated with biological yield. Seed transcript levels of *BnaFAX1-1* were higher in a cultivar with high seed oil content relative to a low-oil cultivar. BnaFAX1-1 localized to the plastid envelope. When expressed in *Arabidopsis thaliana*, *BnaFAX1-1* enhanced biological yield (total plant dry matter), seed yield and seed oil content per plant. Likewise, in the field, *B. napus* *BnaFAX1-1* overexpression lines (*BnaFAX1-1-OE*) displayed significantly enhanced biological yield, seed yield, and seed oil content compared with the wild type. *BnaFAX1-1* overexpression also up-regulated gibberellic acid 4 (GA4) biosynthesis, which may contribute to biological yield improvement. Furthermore, oleic acid (C18:1) significantly increased in *BnaFAX1-1* overexpression seeds.

Conclusion: Our results indicated that the putative fatty acid exporter *BnaFAX1-1* simultaneously improved seed oil production, oil quality and biological yield in *B. napus*, providing new approaches for future molecular breeding.

Introduction

*Brassica napus* (rapeseed) is an important oilseed crop for edible oils, as the oil extracted from its seeds is rich in highly unsaturated fatty acids. In addition, rapeseed oil constitutes a reasonable substitute for diesel fuel as a renewable energy source due to their chemical similarities and high energy potential (Durrett et al., 2008). Increasing rapeseed oil production per hectare would thus increase edible oil and liquid biofuel production. Indeed, substantial work has been invested into improving *B. napus* seed yield and seed oil content. Similarly, increasing total biological yield of above-ground biomass contributes to increasing seed yield and seed oil production (Lu et al., 2016).

Previous studies revealed that rapeseed seed oil production may be enhanced by overexpressing enzymes or transcription factors involved in various metabolic processes. For example, the overexpression of yeast (*Saccharomyces cerevisiae*) GLYCEROL-3-PHOSPHATE DEHYDROGENASE (GPD1) under the control of a seed-specific promoter raised seed oil content (Vigeolas et al., 2007). Likewise, seed oil content increased in transgenic *Arabidopsis thaliana* plants overexpressing *B. napus* WRINKLED1 (*WRI1*)-like (Liu et al., 2010). The conditional expression of *B. napus* LEAFY COTYLEDON1 (*BnLEC1*) and *LEC1-LIKE* (*BnL1L*) in developing seeds enhanced seed oil content by 2–20% with no detrimental effects on major agronomic traits (Tan et al., 2011). Finally, the overexpression of *B. napus* GROWTH REGULATING FACTOR2 (*GRF2*)-like (*BnGRF2*) improved seed oil production by regulating cell
number and plant photosynthesis (Liu et al., 2012). However, to date, no chloroplast membrane proteins have been characterized that improve both seed oil production and biological yield.

Biological yield reflects the total accumulation of photosynthetic products in plant tissues. In plants, photosynthesis and fatty acid (< C18) biosynthesis take place in chloroplasts (Li-Beisson et al., 2013). A recent study revealed that altering carbon metabolism in the chloroplasts of transgenic tobacco (Nicotiana tabacum) plants with high leaf oil levels caused biological yield and oil production to increase. We speculated that photosynthetic products transported across the chloroplast membranes may be critical for biological yield. We previously conducted a genome wide association study (GWAS) of biological yield with 520 B. napus accessions, and identified 88 single-nucleotide polymorphisms (SNPs) that were significantly associated with this trait (Lu et al., 2016). Few proteins have been reported that localize to the chloroplast envelope and function as transporters regulating carbon metabolism across the chloroplast membranes. In previous work, we showed that Arabidopsis FATTY ACID EXPORTER 1 (AtFAX1) localized to the chloroplast inner envelope and mediated fatty acid export from chloroplasts, an essential step in the biosynthesis of leaf and stem lipids (Li et al., 2015). However, loss of function mutation in AtFAX1 had no effect on seed oil content, seed quality, seed yield, or seed oil production. Therefore, how fatty acids are transported from their site of biosynthesis in the plastids of developing seeds remains unknown.

To determine the contribution of chloroplast envelope proteins to biological yield, which might increase seed oil production in B. napus, we identified genes encoding proteins predicted to localize to the chloroplast envelope that mapped near significant SNPs from our previous GWAS results in B. napus. Notably, BnaFAX1-1, the B. napus ortholog of Arabidopsis FAX1, mapped near a significant SNP associated with biological yield. We propose that BnaFAX1-1 is a fatty acid exporter associated with biological yield in B. napus, based on functional annotation analysis, subcellular localization and transcript levels across various B. napus cultivars. Our results revealed that BnaFAX1-1 significantly contributed to biological yield and improved seed oil production and oil quality. Furthermore, we observed that BnaFAX1-1 may modulate gibberellic acid 4 (GA4) content, offering a potential mechanism for the increase in biological yield. The present study provides an important solution to simultaneously improve biological yield, seed oil content, seed yield and oil quality in B. napus by manipulating a single gene: BnaFAX1-1.

**Results**

Identification of chloroplast membrane-localized proteins potentially contributing to biological yield in B. napus

We previously detected SNPs significantly associated with biological yield during a GWAS of 520 B. napus accessions (Lu et al., 2016). We selected 6,627 candidate genes contained within the intervals surrounding 88 significant quantitative trait loci (QTLs) associated with biological yield trait. Of those, 29 encoded proteins with potential localization to the chloroplast membrane, as determined by GO analysis.
We further narrowed down the list of candidates to potential transporters that may be involved in the export of photosynthetic products. Notably, we identified two genes, \textit{BnaA04g02480D} and \textit{BnaA07g17240D}, that were closely linked with the significant SNP Bn-A07-p12412116 (Lu et al., 2016) and encoded orthologous to the \textit{Arabidopsis} membrane protein FAX1, known to mediate plastid fatty acid export.

We focused on the characterization of these two FAX1 orthologous genes. The FAX protein family consists of seven members in \textit{Arabidopsis}, named AtFAX1–7 (Li et al., 2015). To identify potential FAX orthologues in field mustard (\textit{Brassica rapa}), wild cabbage (\textit{Brassica oleracea}) and \textit{B. napus}, we performed BLAST searches, using the 7 Arabidopsis FAX protein sequences as queries, leading to the identification of 9 putative orthologs each in \textit{B. rapa} (\textit{BraFAX}) and \textit{B. oleracea} (\textit{BolFAX}), and 21 in \textit{B. napus} (\textit{BnaFAX}). The physicochemical characteristics (amino acid number, theoretical isoelectric point (pI) values, relative molecular weight and number of transmembrane domains) for BnaFAX proteins are listed in Table S3.

We generated an unrooted neighbor-joining phylogenetic tree based on the 46 protein sequences of FAX family members (Fig. 1B) and discovered that AtFAX1 and six putative BnaFAX1 members (BnaFAX1-1 to BnaFAX1-6) clustered into one branch. To further characterize the \textit{B. napus} FAX family, we analyzed the chromosomal locations and gene structures of the encoding genes (Figure S1A, S1B) and predicted the conserved motifs of BnaFAX proteins using the MEME program (Figure S1C). Of the six \textit{B. napus} FAX1 members within the same branch as AtFAX1, BnaFAX1-1 (\textit{BnaA07g17240D}) and BnaFAX1-2 (\textit{BnaCnng07490D}) were closest to AtFAX1, as evidenced by their very similar gene structures and conserved protein motifs, suggesting that BnaFAX1-1 and BnaFAX1-2 may share the same functions as AtFAX1.

To determine what effect, if any, the six AtFAX1-like genes had on seed oil content, we analyzed their transcript levels across various tissues in one cultivar with high seed oil contents (H, cultivar name: ZS11) and one with low seed oil content (L, cultivar name: ZY821). BnaFAX1-1 was more highly expressed in the H cultivar relative to the L cultivar in all tissues tested (Fig. 1C). By contrast, BnaFAX1-3 (\textit{BnaA04g02480D}) was barely detectable in either \textit{B. napus} cultivar. Besides, we also observed the expression levels of 6 members of BnaFAX1 in 6 tissues of a pair of high- and low- seed oil content accessions grown in Chongqing (CQ24, CQ45) and Yunnan (YN24, YN45), among which CQ24 (seed oil content about 43%) and YN24 (seed oil content about 45%) are high- seed oil content (H-SOC) accessions, and CQ45 (seed oil content about 35%) and YN45 (seed oil content about 37%) are low- seed oil content (L-SOC) accessions. The result is shown in Figure S2, the expression level of BnaFAX1-1 in H-SOC accessions (CQ24, YN24) is higher than that of L-SOC accessions (CQ45, YN45) in the stem (St), leaf (Le), siliques, and seeds on the main inflorescence of 30 days after flowering (30ZP and 30ZS, respectively) and on the primary branch (30CP and 30CS, respectively) (Figure S2A, S2C). This further confirms the conjecture that BnaFAX1-1 may contribute to the formation of seed high oil content in \textit{B. napus}. Furthermore, to further determine whether BnaFAX1-1, BnaFAX1-2 are conducive to the formation of high biological yield (Fig. 1A), the seedling leaves of four pairs with extremely high- (P281, P542, P125,
P257-HBY) and low-biological yield accessions (P319, P276, P131, P81-LBY) were selected for qRT-PCR analysis, and biological yield dry weight per plant for each accession is shown in Figure S2G. The qRT-PCR results showed that the expression levels of \textit{BnaFAX1-1} and \textit{BnaFAX1-2} in high-biological yield accessions were higher than those in low-biological yield accessions (Figure S2E, S2F), which is consistent with the GWAS analysis result (Fig. 1A). Overall, to further determine whether \textit{BnaFAX1-1} can increase both seed oil content and biological yield, we further characterized the function of \textit{BnaFAX1-1}.

**Subcellular localization and transcript levels of \textit{BnaFAX1-1} in \textit{B. napus}**

To determine the subcellular localization of \textit{BnaFAX1-1} in plant cells, we tagged \textit{BnaFAX1-1} with green fluorescent protein (GFP) and expressed the construct under the control of the constitutive \textit{Cauliflower mosaic virus} (CaMV) 35S promoter (Fig. 2A). We transiently transfected \textit{Arabidopsis} protoplasts with the \textit{BnaFAX1-1-GFP} construct, using \textit{AtFAX1-GFP} as a marker for chloroplast envelopes. We observed a ring of fluorescence at the periphery of chloroplasts, which is consistent with a plastid envelope localization for \textit{BnFAX1-1}, as seen previously with \textit{AtFAX1} (Fig. 2B).

We next measured \textit{BnaFAX1-1} transcript levels of in seven tissues across five stages of development (roots, stems, leaves, flowers, buds, seeds, and silique pericarp after flowering 7, 14, 21, 30, 40 d). We observed the highest expression level for \textit{BnaFAX1-1} in leaves and seeds after 40 days of flowering (Fig. 2C). We had previously determined that \textit{AtFAX1} was mainly expressed in leaves, but not in seeds (Li et al., 2015). This result suggested that \textit{BnaFAX1-1} function may differ from that of \textit{AtFAX1}, which did not contribute to seed oil accumulation in \textit{Arabidopsis}.

**Overexpression of \textit{BnaFAX1-1} in \textit{Arabidopsis} and \textit{B. napus}**

Next, we generated \textit{BnaFAX1-1} overexpression constructs and transformed both \textit{Arabidopsis} (Col-0 accession) and \textit{B. napus} (Westar cultivar). We validated overexpression lines (OE) by RT-qPCR using total RNA extracted from \textit{B. napus} and \textit{Arabidopsis} transgenic individuals with \textit{BnaFAX1-1}-specific primers (Table S4). We selected four homozygous \textit{BnaFAX1-1} overexpressing lines in \textit{Arabidopsis}, named OE/At#1, OE/At#2, OE/At#3, and OE/At#4 (Fig. 3A). Similarly, we obtained four \textit{BnaFAX1-1} overexpressing lines in \textit{B. napus}, named OE#17, OE#19, OE#20 and OE#21 (Fig. 4A).

**Increased biological yield and seed oil production by \textit{BnaFAX1-1} overexpression in \textit{Arabidopsis}**

Next, we phenotyped \textit{Arabidopsis} lines overexpressing \textit{BnaFAX1-1} and compared their growth to wild-type (WT) plants. We noticed that all \textit{Arabidopsis BnaFAX1-1} overexpressing lines were slightly larger and produced more biomass than WT plants (Fig. 3B, Table 1). After reaching reproductive maturity, overexpression lines were significantly bigger than the WT, with thicker inflorescence stalks and more siliques (Fig. 3C, Table 1). A detailed analysis of different tissues and organs in transgenic and WT plants grown for 7 weeks revealed that plant height, rosette fresh and dry weight, fresh and the dry weight of biological yield was significantly higher in overexpressing lines relative to WT plants (Table 1). Likewise, stem fresh weight and stem diameter in the transgenic lines were significantly increased compared to WT.
plants. Furthermore, we observed an increase in seed yield per plant in overexpression lines, largely due to an increase in silique number per plant (Table 1). We also determined the total lipid content of mature seeds, which indicated that overexpression lines accumulated more total lipid content relative to the WT (Fig. 3D). Collectively, these results indicate that the heterologous overexpression of BnaFAX1-1 in Arabidopsis promoted plant growth and development, and led to an increase in seed oil production.

### Table 1

| Traits                        | WT       | OE/At#1 | OE/At#2 | OE/At#3 | OE/At#4 |
|-------------------------------|----------|---------|---------|---------|---------|
| Stem fresh weight (mg/cm)     | 12.57 ± 0.79 | 12.63 ± 2.03 | 15.31 ± 2.00** | 15.9 ± 1.28** | 15.86 ± 2.66** |
| Stem diameter (mm)            | 0.97 ± 0.11 | 1.04 ± 0.08 | 1.16 ± 0.99** | 1.23 ± 0.14** | 1.21 ± 0.08** |
| Plant height (cm)             | 32.6 ± 3.37 | 39.74 ± 2.21** | 38.9 ± 1.13** | 37.75 ± 2.5** | 37.49 ± 2.37** |
| Rosette fresh weight (g)      | 1.09 ± 0.14 | 1.28 ± 0.17* | 1.43 ± 0.3* | 1.58 ± 0.36** | 1.44 ± 0.32* |
| Rosette dry weight (g)        | 0.17 ± 0.02 | 0.18 ± 0.02 | 0.22 ± 0.02** | 0.23 ± 0.05** | 0.23 ± 0.04** |
| Fresh weight of BYAG (g)      | 2.91 ± 0.24 | 4.44 ± 0.48** | 4.20 ± 0.45** | 4.85 ± 0.57** | 4.31 ± 0.43** |
| Dry weight of BYAG (g)        | 0.45 ± 0.05 | 0.65 ± 0.07** | 0.68 ± 0.04** | 0.76 ± 0.10** | 0.65 ± 0.14** |
| Length of siliques (cm)       | 1.6 ± 0.09 | 1.57 ± 0.04 | 1.62 ± 0.09 | 1.67 ± 0.07 | 1.59 ± 0.1 |
| Number of siliques per plant  | 349.7 ± 24.18 | 408.7 ± 32.1** | 416.4 ± 39.05** | 501.9 ± 45.95** | 412.75 ± 57.54* |
| Number of seeds per silique   | 61.06 ± 6.24 | 64.33 ± 3.56 | 60.78 ± 4.93 | 63.33 ± 5.69 | 61.94 ± 6.63 |
| Weight of per 1000 seeds (mg) | 19.15 ± 0.28 | 18.37 ± 0.68 | 18.58 ± 0.11 | 19.12 ± 0.12 | 19.36 ± 0.58 |
| Seed yield per plant (mg)     | 118.5 ± 34 | 179.8 ± 45** | 174.9 ± 54* | 241.7 ± 51** | 172.0 ± 42* |

*P < 0.05, **P < 0.01, Student’s t-test (n = 6–10 ± SD). BYAG: biological yield of above ground organs.

**Increased biological yield, gibberellin and leaf lipid contents in B. napus plants overexpressing BnaFAX1-1**

To test the effect of BnaFAX1-1 overexpression in B. napus on biomass accumulation, we analyzed the growth kinetics of three independent BnaFAX1-1 overexpression lines selected at random (OE#17, OE#19 and OE#21) (Fig. 4A). We grew all plants hydroponically in Hoagland nutrient solution for 32 d. All
*BnaFAX1-1* overexpression lines were larger and produced more biomass than their non-transgenic WT control (Fig. 4B). This increase in leaf biomass was reflected in all phenotypes measured: leaf fresh /dry weight and leaf size (including leaf length, leaf width and leaf area; Fig. 4C, 4D). Compared to the WT, overexpression of *BnFAX1-1* also resulted in a significant increase in total root length, root area, root volume, root fresh and dry weight (Fig. 4D). Overexpression of *BnaFAX1-1* in *B. napus* therefore promoted plant growth.

Toward the identification of the potential mechanism linking FAX1 and *B. napus* growth and biomass improvements, we quantified phytohormone contents in the leaves of two transgenic lines (OE#19 and OE#21) and their WT using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). We observed that gibberellic acid A4 (GA4) accumulated to significantly higher levels in transgenic leaves relative to the WT (Fig. 5A). By contrast, the contents of indole-3-acetic acid (IAA), salicylic acid (SA), and jasmonic acid (JA) were similar in the overexpression lines and the WT (Figure S3). GA4 is a bioactive gibberellin that plays critical roles in plant growth and development (Eriksson et al., 2000). To explore the reason behind the increase in GA4 content in the transgenic lines, we performed transcriptome sequencing from leaves of the transgenic lines (OE#19 and OE#21) and WT. We discovered that the GA4 biosynthetic genes *COPALYL DIPHOSPHATE SYNTHASE* (*CPS*), *KAURENOIC ACID OXIDASES* (*KAOs*) and *GA20 OXIDASE* (*GA20OX*) were more highly expressed in the transgenic lines relative to the WT (Fig. 5B). We validated these results by RT-qPCR (Fig. 5C). These results indicate that overexpression of *BnaFAX1-1* led to up-regulated GA4 biosynthesis, which may in turn contribute to biological yield increase in *B. napus*.

To investigate the consequence of BnaFAX1-1 accumulation in the two selected overexpression lines above on membrane lipid contents, we analyzed lipids from 32-d-old leaves using LC-MS/MS (Fig. 6). We observed a higher lipid content for phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the leaves of OE#19 and OE#21 plants when compared to WT (Fig. 6). These results revealed that BnaFAX1-1 share the same role as AtFAX1 when ectopically expressed in leaves for the regulation of leaf lipid and biomass accumulation.

**BnaFAX1-1 enhances biological yield and seed yield of B. napus plants grown in the field**

To determine if the phenotypes seen in *BnaFAX1-1* overexpression lines extended to the field, we sowed seeds for WT and OE lines in a randomized field plot design using four plots for each OE line and WT. We investigated growth characteristics in plants at flowering stage (grown for 175 d). OE plants were clearly bigger and taller compared to WT, and had produced more leaves on the main stem in the same growth period (Fig. 7A, 7C). In addition, OE plants showed larger leaves at the same position relative to WT plants, which was reflected in increased leaf length, leaf width and leaf area (Fig. 7B, 7C). Lastly, OE lines produced thicker main stems than WT plants; only chlorophyll content and the photosynthetic rate of OE lines were similar to those of the WT (Fig. 7C).

We next harvested mature plants from the field to carry out additional measurements. The *BnaFAX1-1* OE lines were significantly taller than WT plants and bore more effective branches (i.e. branches bearing
seeds) per plant (Fig. 8A, 8D-c). Although we observed no differences in the length of the main inflorescence between the WT and transgenic plants, the OE lines did exhibit more siliques per main inflorescence than in WT plants (Fig. 8B). Similarly, total silique number was significantly greater in all OE lines relative to WT (Fig. 8D-f), as were silique length (Fig. 8C) and number of seeds per silique. Together, these results revealed the greater seed yield per plant and biological yield in all OE plants (Fig. 8D), possibly by increasing the number of effective branches and siliques per plant. We did not observe such phenotypes when we overexpressed AtFAX1 in Arabidopsis in our previous work (Li et al., 2015). Therefore, BnaFAX1-1, unlike AtFAX1, may play a vital role in improving seed yield and biological yield in B. napus.

BnaFAX1-1 enhances B. napus seed oil production and improves oil quality

We examined total lipid content in seeds from OE lines and WT at 30 d and 45 d after flowering, as well as in dry seeds following harvest. Overexpression of BnFAX1-1 in B. napus resulted in an increase in total seed lipid contents at all development stages tested relative to WT (Fig. 9A). We also determined the range of TAG molecular species and total TAG content in BnaFAX1-1 OE lines and WT mature dry seeds. We saw a significant rise in the content of many TAG molecular species (TAG 50:2, 50:3, 52:2, 54:2, 54:3, 54:4, 54:5, 56:2, 56:4) and total TAG content in BnaFAX1-1 OE transgenic plants compared to WT (Fig. 9B, C). In addition, an analysis of fatty acid composition of mature dry seeds grown in the field revealed that oleic acid (C18:1) was significantly increased in the transgenic lines, whereas palmitic acid (C16:0), arachidic acid (C20:0) and eicosenic cis (C20:1) were significantly reduced. Stearic acid (C18:0), linoleic acid (C18:2) and linolenic acid (C18:3) contents were similar in the OE lines and WT (Fig. 9D). These results demonstrated that overexpression of BnaFAX1-1 effectively increased seed oil production and oleic acid content. These results therefore revealed that BnaFAX1-1 may have important application value in B. napus molecular breeding to improve seed oil content, oil quality, seed yield and biological yield.

Discussion

As the third largest source of plant oil after oil palm (Elaeis guineensis) and soybean (Glycine max), rapeseed (Brassica napus) produces approximately 15% of edible vegetable oil worldwide (Gracka et al., 2016; Liu et al., 2016; Lu et al., 2018). Enhancing both seed oil production and seed oil quality is a major goal in B. napus breeding, which can be accomplished by improving seed yield, seed oil content and biological yield, separately or in combination. The present study provides a possible and simple solution to simultaneously improve seed oil production, oil quality, seed yield, seed oil content and biological yield in B. napus by raising the expression levels of a single gene. We identified the potential key gene BnaFAX1-1 through the identification of putative chloroplast-localized transporters whose encoding genes mapped close to significant SNPs from a previous GWAS of 520 B. napus accessions for biological yield. Increasing expression of BnaFAX1-1 improved biological yield and seed oil production, especially oleic acid content, which has not been observed in previous studies.
Chloroplasts are the main site of photosynthesis and plant fatty acid production. Recent studies revealed that altering carbon metabolism in the chloroplasts of transgenic oil crops improved biological yield and seed oil production. We hypothesized that export of photosynthates from the chloroplast may contribute to improving biological yield and seed oil production. We therefore focused here on genes encoding proteins predicted to locate to the chloroplast membrane, leading to a first selection of 29 candidate genes (Table S2). From further analysis of these 29 genes, we identified a gene encoding a putative transporter: *BnaA07g17240D (BnaFAX1-1)*. The *Arabidopsis* ortholog of *BnaFAX1-1* is the putative fatty acid exporter *AtFAX1*, which is crucial for biological yield. Both biological yield and seed oil production increased in 35S:*BnaFAX1-1* transgenic *B. napus* plants grown in the field relative to WT plants, highlighting the potential of this gene to improve seed oil production in *B. napus* (Fig. 4, Fig. 6–7).

Furthermore, we determined that the expression of GA biosynthetic genes was up-regulated, in agreement with the higher GA4 levels measured in the OE lines (Fig. 5). GA biosynthesis is derived from geranylgeranyl diphosphate (GGDP), which is synthesized in the chloroplast. GGDP is then converted to ent-kaurene by ent-COPALYL DIPHOSPHATE SYNTHASE (CPS) and ent-KAURENE SYNTHASE (KS). ent-kaurene is then exported from the chloroplast to complete GA biosynthesis. Fatty acid export may affect carbon metabolism in plastids and further affect ent-kaurene contents or export from plastids. GA biosynthesis was reported to improve plant biological yield (Eriksson et al. 2000). Therefore, *BnaFAX1-1* may indirectly affect GA biosynthesis, resulting in the observed increase in biological yield and seed yield, as seen in *BnaFAX1-1* overexpression lines.

Increasing seed yield and seed oil content are two major approaches to enhance seed oil production. In *B. napus*, seed yield is largely determined by three yield component traits: silique number per plant, seed number per silique, and the weight per thousand seeds (Lu et al., 2017). In the present study, the increase in seed yield measured for *B. napus BnaFAX1-1-0E* lines was mainly due to silique number per plant and seed number per silique, as we detected no differences in the weight per thousand seeds between *BnaFAX1-1-0E* and WT plants (Fig. 8C). Seed oil content is another essential contributor to seed oil production, which constitutes the basis for *B. napus* economic importance. Overexpressing *BnaFAX1-1* in *Arabidopsis* and *B. napus* led to a significant rise in seed oil content relative to WT (Fig. 3D, 9A-9C). Therefore, overexpressing *BnaFAX1-1* had great potential practical value to increase seed oil production in *B. napus*.

In our previous study, *Arabidopsis* lines overexpressing *FAX1* showed a pronounced increase of lipids in flowers and leaves (Li et al., 2015). However, the *fax1* loss of function mutation had no effects on seed oil content or seed yield (Li et al., 2015; Li et al., 2016). In the green alga *Chlamydomonas* (*Chlamydomonas reinhardtii*), the overexpression of either *CrFAX1* or *CrFAX2* resulted in the accumulation of intracellular TAG (Li et al., 2019). Recently, AtFAX2 and AtFAX4 were reported to be seed-specific transporters mediating seed embryo fatty acid export for seed oil content accumulation in *Arabidopsis* (Li et al., 2020). These results reveal that the tissue-specificity of *FAX* expression may contribute to lipid accumulation in specific tissues. Notably, we determined that *BnaFAX1-1* is highly expressed in siliques during the seed-filling stage in a cultivar with high seed oil content, when compared to another cultivar with low seed oil
content, indicating a correlation between BnaFAX1-1 function and the regulation of seed oil content and seed yield (Fig. 1C). The present study reveals that BnaFAX1-1 mainly mediated seed plastid fatty acid export for the accumulation of seed TAG during the seed-filling stage, leading to the measured increase in seed oil content in the overexpression lines. By contrast, AtFAX1 had no effect on seed TAG accumulation in Arabidopsis, as AtFAX1 is expressed at low levels during the seed-filling stage.

Oleic acid (C18:1) is an important unsaturated fatty acid component of B. napus oil with high nutritional value and good thermal stability. Oil with high oleic acid content can reduce the risk of cardiovascular disease in overweight individuals (Rudkowska et al., 2006) and effectively prevent arteriosclerosis (Nicolosi et al., 2004). In addition, it is highly resistant to oxidization and degradation at high temperature and enjoys a long shelf life (Talcott et al., 2005). Such oleic acid-rich oils also emit little to no smoke when heated to high temperatures and reduce cooking times (Miller et al., 1987), display high stability during frying, and imbue frying products with excellent aroma. Aside from cooking applications, oleic acid is also a raw material for biodiesel production (Piazza and Foglia, 2001). In the present study, we determined that oleic acid (C18:1) content significantly increased in BnaFAX1-1-OE seeds, while other plastid-derived fatty acids remained unchanged relative to WT (Fig. 9C). In our previous study, AtFAX1 affected the content of plastid-derived fatty acids in Arabidopsis leaves and flowers, but not in seeds (Li et al., 2015). Arabidopsis ATP-BINDING CASSETTE A9 (ABCA9) mediated fatty acids and acyl-CoA import into the endoplasmic reticulum for TAG accumulation. However, the fatty acid composition and contents were not affected by a loss of function in AtABCA9 (Kim et al., 2013). Our results suggest that BnaFAX1-1 may selectively mediate the export of specific plastid-derived fatty acids, and will be the subject of future work.

In conclusion, we identified a fatty acid export protein, BnaFAX1-1, which mediates fatty acid export from plastids in developing seeds. The overexpression of BnaFAX1-1 significantly upregulated seed oil content, oil quality, seed yield and biological yield in B. napus. BnaFAX1-1 overexpression lines resulted in an up-regulation of GA4 biosynthesis, indicating that BnaFAX1-1 overexpression may influence GA biosynthesis, leading to the observed increase in biological yield and seed oil production. Furthermore, BnaFAX1-1 contributed to the accumulation of oleic acid, an unsaturated fatty acid of high economic value, in seeds, thus improving oil quality. The present study provides an important solution to simultaneously improve rapeseed seed oil production, seed oil content, seed yield, biological yield and seed oil quality by modulating the expression of BnaFAX1-1. We propose that BnaFAX1-1 should be a potential target for B. napus molecular breeding in the future.

**Experimental Procedures**

**Phylogenetic analyses, protein properties and sequence analyses of the FAX family**

We downloaded the sequences of FAX proteins from Arabidopsis (Arabidopsis thaliana), field mustard (Brassica rapa), wild cabbage (Brassica oleracea), and B. napus to generate a on multiple protein sequence alignments using the integrated MUSCLE program in MEGA7.0 (Kumar et al., 2016). We also
generated a phylogenetic tree of the FAX family with MEGA7.0 software using the Neighbor-Joining (NJ) method and bootstrap analysis (1,000 replications). We visualized the phylogenetic trees in FigTree v1.4.2 (Aili et al., 2014). We deduced the physical properties of BnaFAX proteins (molecular weight [MW] and isoelectric point [pI]) using the online proteomics database ExPASy (http://web.expasy.org/peptide_mass/) (Gasteiger et al., 2003). We predicted transmembrane domains using the online tool TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Deng et al., 2012). We determined the chromosomal location of all BnaFAX genes by performing Basic Local Alignment Search Tool for Nucleotides (BLASTN) searches of their sequences against the 19 B. napus chromosomes, followed by visualization with the MapChart Software (Voorrips, 2002). We identified exon/intron boundaries for the BnaFAX genes at the Gene Structure Display Server (GSDS 2.0, http://gsds.cbi.pku.edu.cn/index.php) (Guo et al., 2007) and conserved protein motifs with the MEME suite of programs (http://meme-suite.org/) (Bailey et al., 2006), respectively. We then annotated all identified conserved motifs with InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) according to MAST data from the MEME program (Jones et al., 2014).

Vector constructs and plant transformation

We PCR-amplified the complete coding sequence (CDS) of the BnaFAX1-1 gene from B. napus cDNA using the primer pair BnaFAX1-1 FP(XbaI)+ BnaFAX1-1 RP(Sacl) (primers used in this study are listed in Table S4). We then subjected the PCR product to restriction digest with XbaI and SacI and ligated the purified digested product into the pCAMBIA2301M vector (a modified version of pCAMBIA2301 available in our laboratory) to generate pCAMBIA2301-35S:BnaFAX1-1. We introduced the pCAMBIA2301-35S:BnaFAX1-1 construct into Agrobacterium (Agrobacterium tumefaciens) strain GV3101 for transformation of Arabidopsis Col-0 accession by the floral dip method (Clough and Bent, 1998). We selected positive transgenic by germinating seeds on Murashige and Skoog (MS) medium supplemented with 10 mg/L Basta. We also transformed B. napus (Westar cultivar) hypocotyls with Agrobacterium bearing the pCAMBIA2301-35S:BnaFAX1-1 construct based on a protocol described previously (Lu et al., 2013). We selected transgenic calli for growth on MS medium supplemented with Basta, and identified transgenic plants by PCR using BnaFAX1-1-specific primers (F35S3ND+ BnaFAX1-1 RP(SacI), and BnaFAX1-1 FP(XbaI)+NOS5ND) (Table S4). We then transferred positive 35S:BnaFAX1-1 transgenic plants to soil for seed setting and phenotyping.

Subcellular localization of BnaFAX1-1-GFP fusion protein

We PCR-amplified the Arabidopsis FAX1 CDS from Arabidopsis Col-0 accession cDNA using the primer pair AtFAX1 FP(Spel)+ AtFAX1 RP(BamHI) (Table S4). Similarly, we amplified the BnaFAX1-1 CDS by PCR from B. napus cDNA using the primer pair BnaFAX1-1 FP(Spel)+ BnaFAX1-1 RP(BamHI) (Table S4). We subjected both PCR products to restriction digest with SpeI and BamHI before ligation into the pAN580 vector to generate pAN580-35S:AtFAX1-GFP and pAN580-35S:BnaFAX1-1-GFP (Figure2A) (Du et al., 2019), which were used to transiently transfect Arabidopsis protoplasts. We performed the transformation and analysis of Arabidopsis mesophyll protoplasts as described previously (Duy et al.,
GFP fluorescence was detected at 672 to 750 nm and chlorophyll autofluorescence was monitored at 503 to 542 nm by laser scanning confocal microscopy (Carl Zeiss, LSM800). We used 35S:AtFAX1-GFP as a positive control for the chloroplast inner envelope.

**Plant growth conditions and trait measurements**

For *Arabidopsis*, we surface-sterilized seeds from Col-0 and homozygous lines overexpressing *BnFAX1-1* (*BnaFAX1-1-OE*) and sowed them on half-strength MS medium agar plates supplemented with 1% sucrose. We incubated plates in the dark at 4°C for 2 d before releasing them in a plant incubator set to a light/dark photoperiod of 16 h light/8 h dark. After 7 d, we transferred seedlings to soil and allowed them to grow in the incubator under the same conditions. After 7 weeks, we measured a number of phenotypes on all plants: stem fresh weight (mg/cm; 1 cm from the bottom of 2nd internode of the primary inflorescence stem), stem diameter (mm; from bottom part of 2nd internode of the primary inflorescence stem), silique length, plant height, rosette fresh/dry weight, and fresh/dry weight of biological yield above ground per plant. We also collected the number of siliques per plant, the number of seeds per silique, the weight of 1,000 seeds and the seed yield per plant after seeds maturation.

For *B. napus* growth in hydroponics, we germinated seeds for *B. napus* WT (*Westar* cultivar) and BnaFAX1-1-OE lines in glassware covered with three layers of wet filter paper. We transferred 8-d-old seedlings to quarter-strength Hoagland nutrient solution in a plant incubator under a temperature cycle of 26°C (day)/22°C (night) and a photoperiod of 16h light/8h dark. We replaced the nutrient solution with half-strength Hoagland nutrient solution after 5 d, which we replaced 5 d later for full-strength Hoagland nutrient solution. We then refreshed the nutrient solution with full-strength Hoagland nutrient solution every 5 d until harvesting. After 32 d, we measured total root length, root area, root volume, root fresh/dry weight, leaf length, leaf width, leaf area, and leaf fresh/dry weight.

For field experimental work, we sowed *B. napus* WT (*Westar* cultivar) and BnaFAX1-1-OE lines in soil in Beibei, Chongqing (29°45′N, 106°22′E, 238.57 m). Plants grown in field were arranged in a randomized field plot design with three replicates per genotype. Each plot contained four rows: 10 plants per row, 30 cm between rows, and 20 cm between plants within each row. After 175 d, we measured plant height, the number of leaves on the main stem for each plant, leaf length, leaf width, leaf area, the diameter at the same position on the main stem, chlorophyll content and photosynthetic rate for all plants. After seeds maturation, we scored plant height, effective branch number per plant (i.e. branches bearing seeds), length of the main inflorescence, silique number per main inflorescence, silique number per plant, silique length, seeds per silique, weight per 1,000 seeds, overall seed yield per plant, and dry weight of biological yield above ground. We harvested 8-15 plants from each plot, averaged the measurements in each plot and used the resulting data for ANOVA.

**Plant phytohormone extraction**

We harvested 50–75 mg of leaf tissue from 35-d-old *B. napus* WT (*Westar* cultivar) and BnaFAX1-1-OE lines grown in incubator and extracted phytohormones using previously published methods (Pan et al., 2007).
2010). Briefly, we mixed samples in 500–750 μL 2-propanol/water/concentrated hydrochloric acid (2: 1: 0.002 v/v/v) for extraction and it was shaken at 100rpm for 30min at 4°C. We then added 1mL dichloromethane, and it also was shaken at 100rpm for 30min at 4°C, then centrifuged the mixture and collected the supernatant. We repeated this extraction procedure three times, combined all supernatants and dried them under nitrogen flow. Finally, we added 200 μL of methanol/0.1% formic acid aqueous solution (1: 1 v/v) to resuspend the pellet, filtered it through an organic filter and placed each sample into the injection tube. We analyzed phytohormones by liquid chromatography followed by tandem mass spectrometry (LC-MS/SM) (QTRAP 6500+) using the MRM approach described by Pan et al (2010). Briefly, LC uses a binary solvent system. The mobile phase is methanol and 0.05% formic acid. We selected an Eclipse plus C18 (5μm, 2.1 * 150 mm) chromatographic column. The flow rate was controlled at 300 μL / min and the column temperature was 30°C, 10 μL per injection. We used a gradient elution, with the initial gradient of methanol of 10%, held for 2 min, and gradually increased to 10 min and maintained at 90% for 5 min. At 15.1 min, we reduced methanol to the initial gradient and held for 7 min. We added external phytohormone standards for gibberellic acid, abscisic acid, indole-3-acetic acid, salicylic acid and jasmonic acid to calculate the level of each phytohormone in the samples.

**Lipid analyses**

We extracted lipids from 32-d-old leaves and analyzed lipid content by LC-MS/MS using the method reported previously (Lu et al. 2018). We determined the levels and molecular species for phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), diacylglycerol (DAG) and triacylglycerol (TAG) as previously described (Welti et al., 2002). For each experiment, we sampled six plants in six technical replicates; we used 50–100 mg fresh leaf tissue to extract lipids.

We quantified fatty acid composition using a gas chromatograph coupled with a flame ionization detector (GC-FID). We immersed 3-5mg dry seed samples in 1.5 mL methanol containing 1.5% H₂SO₄ and 0.01% BHT. We then added 25 μL heptadecanoic acid triglyceride (C17: 0 TAG) with 5.4 μmol/L in tubes as internal standard. We incubated the tubes in a water bath at 90°C for 1 h before allowing them to cool to room temperature. We then added 1 mL H₂O and 1 mL chromatographic hexane, mixed well, and centrifuged the samples at 1,000 rpm for 10 min. Subsequently, we transferred 0.8 mL of the upper phase to a new glass tube and dried it under nitrogen flow. Finally, we added 0.4 mL hexane to dissolve fatty acid methyl esters, and injected 1 μL of the ester solution into the GC with the detector temperature set to 280°C, oven temperature to 170°C for 2 min, and then increased by 3°C /min up to 210°C.

We determined total lipid content in seeds using a previously described method (Ma et al., 2013). Briefly, we immersed 50 mg dry seeds in 1 mL methanol (chromatography grade) and 2 mL 2% (m/v) NaOH in glass tubes, after which we mashed the seeds with glass rods and vortexed for 10 min on a vortex shaker. Next, we placed the glass tubes containing the seed mixture in a 60°C water bath for 1 h before allowing the tubes to cool to room temperature. We then added 2 mL chloroform (chromatography grade) and vortexed again for 10 min. After centrifugation of the mixture at 5000g for 5 min at room temperature, we
transferred the lower phase (chloroform) to a dry weighed glass tube, added 1mL hexane (chromatography grade) to the remaining upper layer and vortexed again for 10 min. After centrifugation, we collected the upper phase (hexane) and added it to the transferred chloroform solution. Finally, the mixture was dried under nitrogen flow and the content of total lipid was calculated. Five biological replicates were performed for each line.

**RNA Isolation and Quantitative RT-PCR analysis**

We extracted total RNA from various tissues of *B. napus* cultivar Zhongshuang11 (ZS11) using the EZ-10 DNAaway RNA Mini-prep Kit (Sangon Biotech (Shanghai), Co., Ltd). We then synthesized first-strand cDNAs from 1µg total RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. We performed real-time quantitative PCR analysis using SYBR Premix Ex Taq II (Perfect Real Time) (TaKaRa, Dalian, China) in a CFX96 real-time PCR system (Bio-Rad, USA) according to previous methods (Lu et al., 2015). Gene-specific primers were designed using Vector NTI software, with the *BnACTIN7* gene as internal reference gene (Table S4) (Deng et al., 2016). We performed RT-qPCR on three independent biological replicates, each consisting of three technical replicates. We determined *BnaFAX1-1* transcript levels in WT plants and transgenic lines by RT-qPCR as described above. In *Arabidopsis*, we used *ACTIN2* as an internal control (Table S4) (Wei et al., 2012).

**Statistical analysis**

All data were analyzed for statistical significance using SPSS 19.0 and GraphPad Prism 7. Analysis of variance was performed on data sets, and the values are presented as means±SD. We used Duncan's test or Student's *t*-test to analyze the statistical significance between WT and transgenic lines (*P* < 0.05; **P** < 0.01).

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and material**

All data generated or analysed during this study are included in this published article and its supplementary information files.
Competing interests

The authors declare that the research was conducted in the absence of commercial or financial ties that could lead to any conflict of interest

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Author contributions

NNL, JNL and LG conceived and designed the experiments. ZCX, FT, LYZ, STL, SFW, QH, BY, CZ, LJW, CMQ, LG and KL performed the experiments and analyzed the data. ZCX and NNL wrote the paper. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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References

1. Aili SR, Touchard A, Escoubas P, Padula MP, Orivel J, Dejean A, and Nicholson GM. Diversity of peptide toxins from stinging ant venoms. Toxicon. 2014;92:166–78.
2. Bailey TL, Williams N, Misleh C, and Li WW. MEME: discovering and analyzing DNA and protein sequence motifs. Nucleic Acids Res. 2006;34:W369–73.
3. Clough SJ, and Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 1998;16:735–43.
4. Deng XD, Gu B, Li YJ, Hu XW, Guo JC, and Fei XW. The roles of acyl-CoA: diacylglycerol acyltransferase 2 genes in the biosynthesis of triacylglycerols by the green algae Chlamydomonas reinhardtii. Mol Plant. 2012;5:945–7.
5. Deng Z, Li X, Wang Z, Jiang Y, Wan L, Dong F, Chen F, Hong D, and Yang G. Map-based cloning reveals the complex organization of the BnRf locus and leads to the identification of BnRf(b), a male sterility gene, in Brassica napus. Theor Appl Genet. 2016;129:53–64.
6. Du D, Liu M, Xing Y, Chen X, Zhang Y, Zhu M, Lu X, Zhang Q, Ling Y, Sang X, Li Y, Zhang C, and He G. Semi-dominant mutation in the cysteine-rich receptor-like kinase gene, ALS1, conducts constitutive defence response in rice. Plant Biol (Stuttg). 2019;21:25–34.
7. Durrett TP, Benning C, and Ohlrogge J. Plant triacylglycerols as feedstocks for the production of biofuels. Plant J. 2008;54:593–607.
8. Duy D, Wanner G, Meda AR. von Wieren, N., Soll, J., and Philippar, K. (2007). PIC1, an ancient permease in Arabidopsis chloroplasts, mediates iron transport. Plant Cell 19: 986–1006.
9. Eriksson ME, Israelsson M, Olsson O, and Moritz T. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. Nat Biotechnol. 2000;18:784–8.
10. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, and Bairoch A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 2003;31:3784–8.
11. Gracka A, Jelen HH, Majcher M, Siger A, and Kaczmarek A. Flavoromics approach in monitoring changes in volatile compounds of virgin rapeseed oil caused by seed roasting. J Chromatogr A. 2016;1428:292–304.
12. Guo AY, Zhu QH, Chen X, and Luo JC. [GSDS: a gene structure display server]. Yi Chuan. 2007;29:1023–6.
13. Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, McWilliam H, Maslen J, Mitchell A, Nuka G, Pesseat S, Quinn AF, Sangrador-Vegas A, Scheremetjew M, Yong SY, Lopez R, and Hunter S. InterProScan 5: genome-scale protein function classification. Bioinformatics. 2014;30:1236–40.
14. Kim S, Yamaoka Y, Ono H, Kim H, Shim D, Maeshima M, Martinoia E, Cahoon EB, Nishida I, and Lee Y. AtABCA9 transporter supplies fatty acids for lipid synthesis to the endoplasmic reticulum. Proc Natl Acad Sci U S A. 2013;110:773–8.
15. Kumar S, Stecher G, and Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016;33:1870–4.
16. Li-Beisson Y, Shorros B, Beisson F, Andersson MX, Arondel V, Bates PD, Baud S, Bird D, Debono A, Durrett TP, Franke RB, Graham IA, Katayama K, Kelly AA, Larson T, Markham JE, Miquel M, Molina I, Nishida I, Rowland O, Samuels L, Schmid KM, Wada H, Welti R, Xu C, Zallot, R, and Ohlrogge, J. (2013). Acyl-lipid metabolism. Arabidopsis Book 11: e0161.
17. Li N, Gugel IL, Giavalisco P, Zeisler V, Schreiber L, Soll J, and Philippar K. (2015). FAX1, a Novel Membrane Protein Mediating Plastid Fatty Acid Export. Plos Biology 13.
18. Li N, Meng H, Li S, Zhang Z, Zhao X, Wang S, Liu A, Li Q, Song Q, Li X, Guo L, Li H, Zuo J, and Luo K. Two Plastid Fatty Acid Exporters Contribute to Seed Oil Accumulation in Arabidopsis. Plant Physiol. 2020;182:1910–9.
19. Li NN, Xu CC, Li-Beisson YH, and Philippar K. Fatty Acid and Lipid Transport in Plant Cells. Trends Plant Sci. 2016;21:145–58.
20. Li NN, Zhang Y, Meng HJ, Li ST, Wang SF, Xiao ZC, Chang P, Zhang XH, Li Q, Guo L, Igarashi Y, and Luo F. (2019). Characterization of Fatty Acid Exporters involved in fatty acid transport for oil accumulation in the green alga Chlamydomonas reinhardtii. Biotechnology for Biofuels 12.
21. Liu J, Hua W, Yang HL, Zhan GM, Li RJ, Deng LB, Wang XF, Liu GH, and Wang HZ. The BnGRF2 gene (GRF2-like gene from Brassica napus) enhances seed oil production through regulating cell number and plant photosynthesis. J Exp Bot. 2012;63:3727–40.
22. Liu J, Hua W, Zhan G, Wei F, Wang X, Liu G, and Wang H. Increasing seed mass and oil content in transgenic Arabidopsis by the overexpression of wri1-like gene from Brassica napus. Plant Physiol Biochem. 2010;48:9–15.

23. Liu S, Fan C, Li J, Cai G, Yang Q, Wu J, Yi X, Zhang C, and Zhou Y. A genome-wide association study reveals novel elite allelic variations in seed oil content of Brassica napus. Theor Appl Genet. 2016;129:1203–15.

24. Lu K, Guo W, Lu J, Yu H, Qu C, Tang Z, Li J, Chai Y, and Liang Y. Genome-Wide Survey and Expression Profile Analysis of the Mitogen-Activated Protein Kinase (MAPK) Gene Family in Brassica rapa. PLoS One. 2015;10:e0132051.

25. Lu K, Peng L, Zhang C, Lu JH, Yang B, Xiao ZC, Liang Y, Xu XF, Qu CM, Zhang K, Liu LZ, Zhu QL, Fu ML, Yuan XY, and Li JN. (2017). Genome-Wide Association and Transcriptome Analyses Reveal Candidate Genes Underlying Yield-determining Traits in Brassica napus. Frontiers in Plant Science 8.

26. Lu K, Xiao Z, Jian H, Peng L, Qu C, Fu M, He B, Tie L, Liang Y, Xu X, and Li J. A combination of genome-wide association and transcriptome analysis reveals candidate genes controlling harvest index-related traits in Brassica napus. Sci Rep. 2016;6:36452.

27. Lu S, Sturtevant D, Aziz M, Jin C, Li Q, Chapman KD, and Guo L. Spatial analysis of lipid metabolites and expressed genes reveals tissue-specific heterogeneity of lipid metabolism in high- and low-oil Brassica napus L. seeds. Plant J. 2018;94:915–32.

28. Lu SP, Bahn SC, Qu G, Qin HY, Hong Y, Xu QP, Zhou YM, Hong YY, and Wang XM. Increased expression of phospholipase D1 in guard cells decreases water loss with improved seed production under drought in Brassica napus. Plant Biotechnol J. 2013;11:380–9.

29. Ma YB, Wang ZY, Zhu M, Yu CJ, Cao YP, Zhang DY, and Zhou GK. Increased lipid productivity and TAG content in Nannochloropsis by heavy-ion irradiation mutagenesis. Biores Technol. 2013;136:360–7.

30. Miller JF, Zimmerman DC, and Vick BA. Genetic-Control of High Oleic-Acid Content in Sunflower Oil. Crop Sci. 1987;27:923–6.

31. Nicolosi RJ, Woolfrey B, Wilson TA, Scollin P, Handelman G, and Fisher R. Decreased aortic early atherosclerosis and associated risk factors in hypercholesterolemic hamsters fed a high- or mid-oleic acid oil compared to a high-linoleic acid oil. J Nutr Biochem. 2004;15:540–7.

32. Pan X, Welti R, and Wang X. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. Nat Protoc. 2010;5:986–92.

33. Piazza GJ, and Foglia TA. Rapeseed oil for oleochemical usage. Eur J Lipid Sci Tech. 2001;103:450–4.

34. Rudkowska I, Roynette CE, Nakhasi DK, and Jones PJ. Phytosterols mixed with medium-chain triglycerides and high-oleic canola oil decrease plasma lipids in overweight men. Metabolism. 2006;55:391–5.

35. Talcott ST, Duncan CE, Pozo-Insfran D, and Gorbet DW. Polyphenolic and antioxidant changes during storage of normal, mid, and high oleic acid peanuts. Food Chem. 2005;89:77–84.
36. Tan H, Yang X, Zhang F, Zheng X, Qu C, Mu J, Fu F, Li J, Guan R, Zhang H, Wang G, and Zuo J. Enhanced seed oil production in canola by conditional expression of Brassica napus LEAFY COTYLEDON1 and LEC1-LIKE in developing seeds. Plant Physiol. 2011;156:1577–88.

37. Vigeolas H, Waldeck P, Zank T, and Geigenberger P. Increasing seed oil content in oil-seed rape (Brassica napus L.) by over-expression of a yeast glycerol-3-phosphate dehydrogenase under the control of a seed-specific promoter. Plant Biotechnol J. 2007;5:431–41.

38. Voorrips RE. MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered. 2002;93:77–8.

39. Wei Q, Hu P, and Kuai BK. Ectopic expression of an Ammopiptanthus mongolicus H+-pyrophosphatase gene enhances drought and salt tolerance in Arabidopsis. Plant Cell Tissue Organ Culture. 2012;110:359–69.

40. Welti R, Li WQ, Li MY, Sang YM, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, and Wang XM. Profiling membrane lipids in plant stress responses - Role of phospholipase D alpha in freezing-induced lipid changes in Arabidopsis. Journal Of Biological Chemistry. 2002;277:31994–2002.

**Figures**
Figure 1

Identification and analysis of new FAX genes in B. napus (A) We selected genes encoding chloroplast membrane proteins based on significant single-nucleotide polymorphisms (SNPs) associated with biological yield in rapeseed (13/14CQ-BY: 2013/2014Chongqing-biological yield). BnaFAX1-1 (BnaA07g17240D) was closely linked to the significant SNP Bn-A07-p12412116 for biological yield, according to our previous work (Lu et al., 2016). (B) Phylogenetic analysis of FAXs in B. napus, B. rapa, B. oleracea, and A. thaliana. (C) Expression analysis of BnaFAX1 in different tissues collected from high-oil content (ZS11-HO) and low-oil content (ZY821-LO) B. napus genotypes. Student’s t-test (n=3-5±SD). Ro: Root, St: stem, Le: leaf, FL: flower, SP: siliques pericarp, SP7D: siliques pericarp after flowering 7d, Se: seeds, Se7D: seeds after flowering 7d.
Figure 2

Subcellular localization of BnaFAX1-1 and expression analysis of BnaFAX1-1 in B. napus (A) Schematics of the transgene cassette bearing 35S:BnaFAX1-1 in a modified binary vector. (B) Localization of the green fluorescent protein (GFP) -FAX1-1 protein. We transiently transfected Arabidopsis leaf protoplasts with constructs encoding GFP-tagged Arabidopsis FAX1 and B. napus FAX1-1. (C) Expression pattern of
BnaFAX1-1 in different tissues in *B. napus* cultivar ZS11. *P<0.05, **P<0.01, Student's t-test (n=4–6; data are means ±SD).

**Figure 3**

Phenotypic analysis of *Arabidopsis* lines overexpressing BnaFAX1-1 (A) Relative expression levels of BnaFAX1-1 in *Arabidopsis* overexpressing lines. (B, C) Growth characteristics of 30-d-old (B) and 49-d-old plants (C) grown in incubators. (D) Total lipid content and fatty acid composition in mature *Arabidopsis* seeds. *P<0.05, **P<0.01, Student's t-test (n=4–6±SD).
Figure 4

Growth and phenotypic analysis of B. napus lines overexpressing BnaFAX1-1 in the plant incubator (A) Relative expression levels of BnaFAX1-1 in leaves from WT and transgenic lines. BnACTIN7 was used as the internal reference. (B) Representative photographs of plants from WT and transgenic lines grown in the plant incubator for 32 d. (C) Representative photographs of the third and fourth leaves of 32-d-old plants. (D) Phenotypic analysis of WT and transgenic plants in the plant incubator. *P<0.05, **P<0.01, Student’s t-test (n=8±SD).
Figure 5

Overexpression of BnaFAX1-1 in B. napus upregulates gibberellic acid biosynthesis, which may contribute to biological yield increase (A) GA4 (gibberellic acid A4) content in leaves from 32-d-old WT and B. napus lines overexpressing BnaFAX1-1. (B) Expression patterns of GA4 biosynthetic and metabolic genes. The yellow indicates higher gene expression levels and the blue indicates lower gene expression levels. COPALYL DIPHOSPHATE SYNTHASE, CPS; KAURENE SYNTHASE, KS; KAURENE OXIDASE, KO; KAURENOIC ACID OXIDASES, KAOS; GA 20-OXIDASE, GA20OX; GA 2-OXIDASE, GA2OX; GA 3-OXIDASE, GA3OX; GIBBERELLIC ACID CARBOXYL METHYLTRANSFERASE, GAMT. (C) Confirmation of the relative expression levels of selected genes by RT-qPCR. *P<0.05, **P<0.01, Student’s t-test (n=4–6±SD).
Figure 6

Accumulation of major polar membrane lipids species in leaves from 32-d-old WT and lines overexpressing BnaFAX1-1 Abbreviations: phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylglycerol, PG; phosphatidylinositol, PI; phosphatidylserine, PS; phasphatidic acid, PA; diacylglycerol, DAG; triacylglycerol, TAG. *P<0.05, **P<0.01, Student's t test (n=4–6±SD).
Figure 7

Growth of B. napus lines overexpressing BnaFAX1-1 in the field (A) Representative photographs of 175-d-old plants grown in the field. (B) Representative photographs of leaves at the same position. (C) Summary of phenotypic analysis of WT and B. napus lines overexpressing BnaFAX1-1 in the field. Plant height, a; plant leaf number, b; leaf length, c; leaf width, d; leaf area, e; upper stem diameter, f; middle stem diameter, g; lower stem diameter, h; chlorophyll content i; photosynthetic rate, j. *P<0.05, **P<0.01, Student’s t-test (n=10±SD).
Figure 8

Phenotypes of B. napus lines overexpressing BnaFAX1-1 after harvest in the field (A) Representative photographs of mature plant at harvest. (B) Silique per main inflorescence from mature plants at harvest and statistic of the silique number per main inflorescence. (C) Simplified diagram of Brassica napus and statistic of siliques length. (D) Summary of phenotypic analysis of B. napus WT and lines overexpressing BnaFAX1-1 in the field. Plant height, a; main inflorescence length, b; effective branch number (seed-bearing), c; siliques number per plant, d; seed number per siliques, e; weight per 1000 seeds, f; seed yield per plant, g; biological yield above ground, h. *P<0.05, **P<0.01, Student’s t-test (n=10±SD).
Figure 9

Analysis of total lipids, TAG content and fatty acid composition in B. napus WT and lines overexpressing BnaFAX1-1 (A) Total lipid content of seeds at different development stages in WT and in lines overexpressing BnaFAX1-1. *P<0.05, **P<0.01, Student's t-test (n=4–6±SD). (B, C) Accumulation of the TAG molecular species (B) and total TAG content (C). (D) Analysis of fatty acid composition. *P<0.05, **P<0.01, Student's t-test (n=6±SD).
Supplementary Files

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