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ORIGINAL ARTICLE

Genome-Wide Identification and Comparative Expression Profile Analysis of the Long-Chain Acyl-CoA synthetase (*LACS*) Gene Family in Two Different Oil Content Cultivars of *Brassica napus*

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Abstract

Long-chain acyl-CoA synthetase (LACS) is one of the key enzymes involved in fatty acid metabolism, including phospholipid biosynthesis, triacylglycerol (TAG) biosynthesis, and fatty acid β-oxidation in plants. However, the characterization of LACSs family in seed oil biosynthesis of *Brassica napus* (*B. napus*) remains unknown. In the present study, we performed a comprehensive genome-wide analysis of this gene family in *B. napus,* and 34 *B. napus LACS* genes (*BnaLACSs*) were identified. Phylogenetic analysis classified the BnaLACS proteins into four groups (A, B, C, and D), which were supported by highly conserved gene structures and consensus motifs. RNA-Sequencing (RNA-Seq) and qRT-PCR combined analysis revealed that 18 *BnaLACSs* (*BnaLACS1-2*, *1–3*, *1–4*, *1–9*, *1–10*, *2–1*, *2–2*, *4–1*, *4–2*, *6–1*, *6–2*, *6–4*, *7–1*, *7–2*, *8–1*, *8–2*, *9–3*, and *9–4*) were highly expressed in developmental seeds. Comparative expression analysis between extremely high oil content (P1-HO) and low oil content (P2-LO) *B. napus* cultivars revealed that *BnaLACS6-4*, *BnaLACS9-3*, and *BnaLACS9-4* may be involved in fatty acid synthesis in chloroplast, and *BnaLACS1-10* and *4–1* may play a vital role in lipid biosynthesis in *B. napus,* which is important for further seed oil accumulation in oilseed rape. The present study provides important information for functional characterization of *BnaLACSs* in seed oil metabolism in *B. napus*.

Keyword *Brassica napus.* · Long-chain acyl-CoA synthetases · Phylogenetic analysis · RNA-Seq · qRT-PCR · Expression analysis

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Introduction

Fatty acids (FAs) are not only essential for membrane constituents, but also are necessary for plant growth, development, and performance. In the process of fatty acid biosynthesis, long-chain acyl groups (C16-ACP and C18-ACP) were synthesized by sequential condensation of two-carbon units in chloroplasts, and then they can be hydrolyzed by fatty acyl thioesterase A/B (FATA/B) to release free fatty acids which are eventually activated to form acyl-CoA esters by a long-chain acyl-CoA synthetase (LACS) and then exported to the endoplasmic reticulum for generating triacylglycerols (TAGs) (Li-Beisson et al. 2010). Therefore, LACSs play pivotal roles in fatty acid (FA) metabolism (Groot et al. 1976; Tan et al. 2014).

In previous studies, the role of LACSs during lipid biosynthesis was well studied in bacteria (Overath et al. 1969; Black et al. 1992), yeast (*Saccharomyces cerevisiae*) (Black and DiRusso 2007), green algae (*Chlamydomonas reinhardtii*) (Jia et al. 2016), mammalian cells (Jetter and Kunst 2008), and some plants (e.g., *Arabidopsis thaliana*) (Shockey et al. 2002; Pulsifer et al. 2012). In *A. thaliana*, nine *LACS* genes make up the LACS family (*AtLACSs*), which participated in the biosynthetic pathways of many fatty acid-derived molecules and were localized in different cellular organelles, such as *AtLACS9* was located at the plastid for TAG formation (Shockey et al. 2002; Weng et al. 2010; Zhao et al. 2010); *AtLACS1*-*AtLACS3* involved in aid transmembrane movement of fatty acids and cuticle biosynthesis (Schnurr et al. 2004; Lu et al. 2009; Pulsifer et al. 2012). It was suggested that members of *LACS* gene family were functionally redundant in lipid metabolism. For example, *AtLACS1* partially overlapped with that of *AtLACS9* in seed oil biosynthesis (Schnurr et al. 2002; Zhao et al. 2010); *AtLACS4* and *AtLACS9* have a strong overlapping function in lipids trafficking between ER and plastid (Jessen et al. 2015). Moreover, the *AtLACSs* tend to have a non-conservative and tissue-specific expression pattern, such as *AtLACS1*, *AtLACS2*, *AtLACS4*, and *AtLACS9*, and showed higher expression levels in developing seeds, whereas *AtLACS5* was only expressed in flowers (Shockey et al. 2002). Similarly, homologs of *LACS* were also well proved to have key roles in fatty acid (FA) metabolism. In cotton, *GhACS1* and *GhACS2* were proved to be anther-specific and *GhACS1* was a prerequisite for normal microsporogenesis in early anther development (Wang and Li 2009). In soybean, *GmLACS* was required for the synthesis of major fatty acids, such as linoleic acid (C-18:3) and oleic acid (C-18:1) (Jang et al. 2015). In *Ricinus communis*, *RcLACS2* could preferentially activate ricinoleate to its CoA thioester to be used for synthesis of TAG (He et al. 2007).

Brassica napus (*B. napus*) is a worldwide oil crop that can be used for direct human consumption and as a source of biofuel, and thereby is regarded as a promising renewable energy crop (Jian et al. 2016). As compared to Canada and Europe (45–50%), the oil content of *B. napus* is about 40% in the main producing area of the Yangtze River Basin of China. Therefore, there is still great potential to improve the oil content of *B. napus* varieties in China. In *B. napus*, two *LACSs* homologous genes (*BnLACS4* and *ACS6*) were isolated and proved to be

involved in lipid biosynthesis and oil accumulation (Pongdontri and Hills 2001; Tan et al. 2014). Despite this, the functions of the whole *LACS* gene family in *B. napus* remain unclear. We are still confused about the roles of *LACSs* in *B. napus*, especially their roles in plastidial fatty acid export for TAG formation. Therefore, a systematic research on the entire *LACS* gene family in *B.napus* is necessary for screening key *Brassica napus LACS* genes (*BnaLACSs*) that play a key role in plastidial fatty acid transport for TAG synthesis. The allotetraploid *B. napus* was formed by the spontaneous hybridization between *B. rapa* and *B. oleracea* approximately 7500 years ago (Chalhoub et al. 2014). Therefore, in the current study, we analyzed the evolutionary relationship of the *BnaLACS* gene family in *B. napus*, *B. rapa*, and *B. oleracea*. Meanwhile, to focus on the *BnaLACS* gene family, two cultivars with extremely different seed oil contents were selected for in-depth analysis.

In the present study, there are two main research objectives. One is to gain a comprehensive understanding about evolutionary relationship of the *LACS* gene family in *Arabidopsis*, *B. napus*, *B. rapa*, and *B. oleracea*. And the other is to obtain an important clue for further studies on the functions of *BnaLACSs* in *B. napus* seed oil accumulation by looking for the *BnaLACS* genes for differential expression in extremely high oil content (P1-HO) and low oil content (P2-LO) *B. napus* cultivars.

Materials and Methods

Identification of LACS Proteins in *B. napus*, *B. rapa*, and *B. oleracea*

Sequences of the nine *Arabidopsis* LACS proteins (AtLACSs) were from the *Arabidopsis* genome database (https://www.Arabidopsis.org/) (Lamesch et al. 2012). To identify the candidate LACS proteins in *B. napus* genome, the BLASTP analysis (Altschul et al. 1997) was carried out using AtLACS proteins as query with a threshold e-value of 1e-20. Similarly, LACSs in *B. rapa* and *B. oleracea* were identified by BLASTP analysis in the BRAD database (https://brassicadb.org/brad/index.php) as well. The coding sequences (CDSs) and genomic sequences of homologous in *B. rapa*, *B. oleracea*, and *B. napus* were obtained from the BRAD (Cheng et al. 2011).

Phylogenetic Analyses of the LACS Families in *B. napus*

To comprehensively understand the evolutionary relationships of LACS family members, a phylogenetic tree was generated based on the multiple alignments of the LACS proteins in *A. thaliana*, *B. rapa*, *B. oleracea*, and *B. napus* by the MEGA7.0 program (Tamura et al. 2011, 2013) using the neighbor-joining (NJ) method. The reliability of the phylogenetic tree was calculated by bootstrap analysis with 1000 replications. Pairwise gap deletion mode was used. The dendrogram was visualized

using the program FigTree v1.4.2 (https://tree.bio.ed.ac.uk/software/figtree/) (Zhu et al. 2015).

Protein Properties and Sequence Analyses

The molecular weight (MW) and isoelectric points (pI) of the candidate LACS proteins were predicted by the online ExPASy proteomics server database (https://web.expasy.org/peptide_mass/) (Gasteiger 2003). The protein tertiary structures were predicted using the online SWISS-MODEL workspace (https://swissmodel.expasy.org/interactive) (Arnold et al. 2006). The Gene Structure Display Server 2.0 (https://gsds.cbi.pku.edu.cn/index.php) was used to conduct exon–intron structure analysis (Guo et al. 2007). The MEME program was applied to identify the conserved motifs (https://meme-suite.org/) (Bailey et al. 2006). Subsequently, all identified conserved domains were annotated using InterProScan (https://www.ebi.ac.uk/interpro/search/sequence-search) (Quevillon et al. 2005). The positional information of all *BnaLACSs* was acquired from *B. napus* Genome Browser database (https://www.genoscope.cns.fr/brassicanapus/). The location images of *BnaLACSs* were then drawn by MapChart Software (Voorrips 2002).

Expression Pattern Analysis of *BnaLACS* Genes

To further characterize the different expression patterns of the *BnaLACSs* in various organs, we analyzed RNA-sequencing (RNA-seq) data. Transcriptome sequencing datasets were deposited in the BioProject ID PRJNA358784, which includes various *B. napus* cultivar ZS11 tissues of roots, stems, leaves, buds, anthocaulus, anthers, sepal, filaments, petal, stamens, pistils, the top of main inflorescence flowers, silique pericarps, seeds, seed coat, and embryos at seedling, budding, initial flowering, and full-bloom stages. The expression levels of all *BnaLACS* genes were quantified based on their fragments per kilobase of exon per million mapped fragments (FPKM) values using Cufflinks with default parameters (Trapnell et al. 2012), and then RNA-seq results were extracted according to their *B. napus* code.

To further confirm the reliability of RNA-seq, the expressions of *BnaLACSs* in different tissues at distinct developmental stages were analyzed by qRT-PCR method. Meanwhile, the expression patterns of *BnaLACSs* in the seed development were also analyzed, using the RNA-seq data in GEO in NCBI (https://www.ncbi.nlm.nih.gov/geo) (accession number GSE77637) (Wan et al. 2016). For RNA-Seq results, $\log_2$ expression values of the 34 *BnaLACS*s were used for heatmap generation using HemI software (Deng et al. 2014). Moreover, 19 *BnaLACSs* were further selected for comparative analyzing their expressions between the high- (P1-HO) and low oil content (P2-LO) *B. napus* cultivars.

Plant Materials Used for Gene Expression Analysis

Seeds of *B. napus* cultivar Zhongshuang 11 (ZS11), SWU47(P1-HO) (approximately 43% oil content in the seeds), and Ningyou12 (P2-LO) (about 33% oil content in the seeds) were provided by Chongqing Engineering Research Center for Rapeseed, China. Plants were cultivated in field conditions at Beibei, Chongqing (29°45′N, 106°22′E, 238.57 m). Each accession contained three rows: 10 plants per row, 30 cm between rows, and 20 cm between plants within each row. All accessions (ZS11, SWU47, and Ningyou12) were sown at the end of September in 2016, and harvested at the beginning of May in the following year. Field fertilization is carried out according to local customs and does not require any chemical treatment. To verify the reliability of RNA-seq in ZS11, tissues of roots (Ro-f), stems (St-f), leaves (Le-f), flowers (Fl-f), buds (Bu-f) in the flowering period; seeds (Se-7d), silique pericarps (SP-7d), seeds (Se-14d), silique pericarps (SP-14d), seeds (Se-21d), silique pericarps (SP-21d), stems (St-30d), seeds (Se-30d), silique pericarps (SP-30d), seeds (Se-40d), and silique pericarps (SP-40d) (7, 14, 21, 30, 40 days after pollination, respectively) were collected. Meanwhile, to survey the expression patterns of *BnaLACSs* in seeds at different developmental stages of P1-HO and P2-LO, seeds on the main inflorescence after pollination at 7 (Se-7d), 14 (Se-14d), 21(Se-21d), 30 (MSe-30d), 40 (Se-40d) days and seeds on the primary branches at 30 (BSe-30d) days were collected, respectively. For each sample, three biological replicates were collected from three independent plants. All samples were immediately frozen in liquid nitrogen and stored at − 80 ℃ until use.

RNA Isolation and Real-Time Quantitative PCR

Total RNA was extracted from all samples with the EZ-10 DNAaway RNA Mini-prep Kit (Sangon Biotech Co., Ltd, Shanghai, China). Then cDNA was synthesized from 1 μg of total RNA using PrimeScript™ RT reagent Kit (Perfect Real Time; TaKaRa Biotechnology, Dalian, China). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Premix Ex Taq II (Perfect Real Time; TaKaRa, Dalian, China) according to the methods described in Lu et al. (Lu et al. 2015). To gain reliable and reproducible results, we performed three technical replicates for each sample. *BnACTIN7* (EV116054) was used as an internal control to evaluate relative gene expression levels (Wu et al. 2010). The values represent the average ± standard error (SE) from the three independent biological replicates. All the qRT-PCR primers are listed in Table S2.

Table 1 A complete list of 34 identified BnaLACSs in our study

Isoforms	Transcript name	At Orthologs	Location	gDNA size (bp)	Exon & Intron	CDS size (nts)	Peptide residues	Theoretical pI	Theoretical Mw (Da)
BnaLACS1-1	BnaAnng37650D	AT2G47240.1	chrAnn_random:42,652,755–42,655,171	2417	6&5	465	154	5.11	16,947.69
BnaLACS1-2	BnaAnng39590D	AT2G47240.1	chrAnn_random:45,115,653–45,116,893	1241	7&6	636	211	5.96	23,214.88
BnaLACS1-3	BnaC04g51420D	AT2G47240.1	chrC04:48,710,206–48,715,999	5794	17&16	1983	660	6.33	74,496.94
BnaLACS1-4	BnaA04g26960D	AT2G47240.1	chrA04:19,042,745–19,049,906	7162	17&16	1983	660	6.01	74,722.1
BnaLACS1-5	BnaC03g25940D	AT2G47240.1	chrC03:14,648,077–14,654,186	6110	20&19	2058	685	8.56	77,815.5
BnaLACS1-6	BnaCnng62450D	AT2G47240.1	chrCnn_random:62,252,868–62,255,208	2341	9&8	1239	412	6.29	46,988.16
BnaLACS1-7	BnaC04g00320D	AT2G47240.1	chrC04:130,463–133,183	2721	10&9	1296	431	6	49,011.27
BnaLACS1-8	BnaA05g00630D	AT2G47240.1	chrA05:304,482–307,202	2721	10&9	1296	431	6	49,011.27
BnaLACS1-9	BnaCnng60230D	AT2G47240.1	chrCnn_random:60,021,103–60,022,674	1572	6&5	855	284	5.7	32,090.3
BnaLACS1-10	BnaA05g00640D	AT2G47240.1	chrA05:369,047–373,989	4943	7&6	882	293	5.38	33,121.48
BnaLACS2-1	BnaA05g16170D	AT1G49430.1	chrA05:10,774,154–10,779,061	4908	19&18	2001	666	5.98	74,373.26
BnaLACS2-2	BnaC05g51350D	AT1G49430.1	chrC05:2,357,024–2,363,526	6503	19&18	2001	666	6.21	74,269.16
BnaLACS3-1	BnaAnng19690D	AT1G64400.1	chrAnn_random:21,545,177–21,551,884	6708	25&24	2883	960	9.09	107,784.56
BnaLACS3-2	BnaC09g11030D	AT1G64400.1	chrC09:7,630,515–7,635,842	5328	21&20	2361	786	6.25	88,342.56
BnaLACS4-1	BnaA01g13470D	AT4G23850.1	chrA01:6,850,542–6,855,021	4480	18&17	2004	667	5.96	74,462.85
BnaLACS4-2	BnaC01g15670D	AT4G23850.1	chrC01:10,752,424–10,756,922	4499	18&17	2004	667	5.96	74,464.89
BnaLACS5-1	BnaC09g26090D	AT4G11030.1	chrC09:25,876,013–25,880,576	4564	19&18	2202	733	5.98	81,506.48
BnaLACS5-2	BnaA09g22150D	AT4G11030.1	chrA09:14,727,049–14,730,917	3869	18&17	2001	666	6.25	74,288.4
BnaLACS5-3	BnaC02g28920D	AT4G11030.1	chrC02:28,320,133–28,324,178	4046	20&19	1986	661	5.96	73,452.51
BnaLACS5-4	BnaA02g21860D	AT4G11030.1	chrA02:14,250,317–14,254,300	3984	20&19	1980	659	6.05	73,395.55
BnaLACS6-1	BnaC03g34500D	AT3G05970.1	chrC03:20,903,411–20,908,669	5259	23&22	2091	696	7.56	76,243.01
BnaLACS6-2	BnaA03g29320D	AT3G05970.1	chrA03:14,247,032–14,252,281	5250	23&22	2091	696	7.56	76,202.95
BnaLACS6-3	BnaC05g45860D	AT3G05970.1	chrC05:41,607,925–41,613,374	5450	23&22	2064	687	7.57	75,318.94
BnaLACS6-4	BnaA05g31340D	AT3G05970.1	chrA05:21,610,343–21,615,685	5343	23&22	2064	687	7.57	75,321.96
BnaLACS6-5	BnaC07g41010D	AT3G05970.1	chrC07:41,067,647–41,068,924	1278	8&7	645	214	9.26	24,024.86
BnaLACS6-6	BnaC07g50530D	AT3G05970.1	chrC07:2,607,718–2,610,857	3140	9&8	705	234	9.21	2546.15
BnaLACS7-1	BnaC07g28060D	AT5G27600.1	chrC07:33,371,838–33,376,153	4316	23&22	2100	699	6.31	77,207.29

Table 1 (continued)

Isoforms	Transcript name	At Orthologs	Location	gDNA size (bp)	Exon & Intron	CDS size (nts)	Peptide residues	Theoretical pI	Theoretical Mw (Da)
BnaLACS7-2	BnaA06g28680D	AT5G27600.1	chrA06:19,660,381–19,664,851	4471	23&22	2100	699	6.72	77,023.14
BnaLACS8-1	BnaC03g44430D	AT2G04350.1	chrC03:29,547,508–29,550,894	3387	11&10	2151	716	8.11	78,022.72
BnaLACS8-2	BnaA03g57930D	AT2G04350.1	chrA03:1,374,321–1,378,991	4671	11&10	2166	721	7.12	78,587.38
BnaLACS9-1	BnaA02g13270D	AT1G77590.1	chrA02:7,290,027–7,291,436	1410	5&4	591	196	9.27	22,368.16
BnaLACS9-2	BnaA10g20970D	AT1G77590.1	chrA10:14,540,117–14,540,680	564	3&2	390	129	9.51	14,939.53
BnaLACS9-3	BnaC06g20910D	AT1G77590.1	chrC06:22,958,082–22,961,638	3557	10&9	2082	693	6.25	75,991.46
BnaLACS9-4	BnaA07g20920D	AT1G77590.1	chrA07:16,300,672–16,304,048	3377	10&9	2082	693	6.25	75,991.46

Results

Identification and Phylogenetic Analysis of *LACSs* in *Brassica napus*

After conducting the local BLASTN and BLASTP analysis, 34, 12, and 16 *LACS* genes were obtained from *B. napus*, *B. rapa*, and *B. oleracea* genomes, respectively. The amino acid (aa) residues in length of the putative 34 BnaLACS proteins varied from 129 (BnaLACS9-3) to 960 (BnaLACS3-1), with an average length of 562 aa. The theoretical pI values ranged from 5.11 (BnaLACS1-1) to 9.51 (BnaLACS9-3), including 22 members exhibiting pI values < 7 (Table 1). Furthermore, the theoretical molecular weight (MW) varied from 2546.15 Da (BnaLACS6-6) to 107,784.56 Da (BnaLACS3-1). Similarly, the *LACS* genes in *B. rapa* and *B. oleracea* genomes are listed in Table S1.

To understand the phylogenetic relationships among LACS proteins from *A. thaliana*, *B. rapa*, *B. oleracea,* and *B. napus*, we constructed an unrooted neighbor-joining (NJ) phylogenetic tree using MEGA7.0 software (Tamura et al. 2011, 2013) based on the multi-alignment of the full-length amino acid sequences. The generated tree was clustered into four groups, termed A, B, C, and D, based on the topology and bootstrap values of the phylogenetic tree (Fig. 1). AtLACS1, ten BnaLACSs, two BraLACSs, and four BolLACSs belonged to the group A. 24 LACS proteins, including ten BnaLACSs, four AtLACSs, five BraLACSs, and five BolLACSs belonged to the group B. Group C consisted of AtLACS6, AtLACS7, eight BnaLACSs, three BraLACSs, and four BolLACSs. AtLACS8, AtLACS9, six BnaLACSs, two BraLACSs, and three BolLACSs formed Group D. These results indicated that the homologous genes of *LACS* underwent a big expansion *in B. napu*s as compared with those in *Arabidopsis*.

Chromosomal Distribution, Genomic Structure, Conserved Motif, and Tertiary Structure of *BnaLACSs*

Chromosomal location analysis showed that the 34 *BnaLACS*s were mapped to 17 of the 19 *B. napus* chromosomes, and the genes were unevenly distributed on almost all chromosomes except for A08 and C08 chromosomes (Fig. S1). Chromosome A05 contained the largest number (4) of *BnaLACS*s, chromosomes C03 and C07 contained three genes; chromosomes A02, A03, C04, C05, and C09 contained two *BnaLACS*s; while only one *BnaLACS* gene was located on chromosomes A01, A04, A06, A07, A09, A10, C01, C02, and C06, respectively (Fig. S1). In addition, three and two *BnaLACS* genes are located on Ann_random and Cnn_random, respectively.

Genomic organization plays an important role in the evolution of a gene family (Xu et al. 2012; Wang et al. 2015). To further examine the exon–intron structure of *BnaLACS*s, the CDSs and their corresponding genomic sequences of *BnaLACS*s were aligned manually. The number of exons showed a high degree of divergence among the 34 *BnaLACS*s, ranging from 3 to 25 (Fig. 2a). However, the *BnaLACS*s within the same group shared similar gene structures, indicating the close

relationship. For instance, almost all genes in Group B had 17 to 21 exons, except *BnaLACS3-1*. Most members (70%) in Group C had 23 exons and genes in Group D had 10 to 11 exons, except *BnaLACS9-1* and *BnaLACS9-2*.

We predicted 20 putative conserved motifs in all the AtLACS and BnaLACS proteins using the MEME program. As shown in Fig. 2b, motifs 2, 10, and 17 were detected in Group A–C. Motifs 1–9, 12, and 18 were observed in many LACS proteins, accounting for 83.7, 88.4, 88.4, 86, 86, 88.4, 81.4, 88.4, 86, 90.7, and 86% of the total LACS proteins investigated, respectively. Motif 15,16, 19, and 20 was only detected in Group C, A, B, and D, respectively. According to InterProScan annotation, motif 4 was an AMP-binding enzyme C-terminal domain, motifs 1, 2, 6, and 11 were AMP-dependent synthetase/ligase. These five motifs are functionally

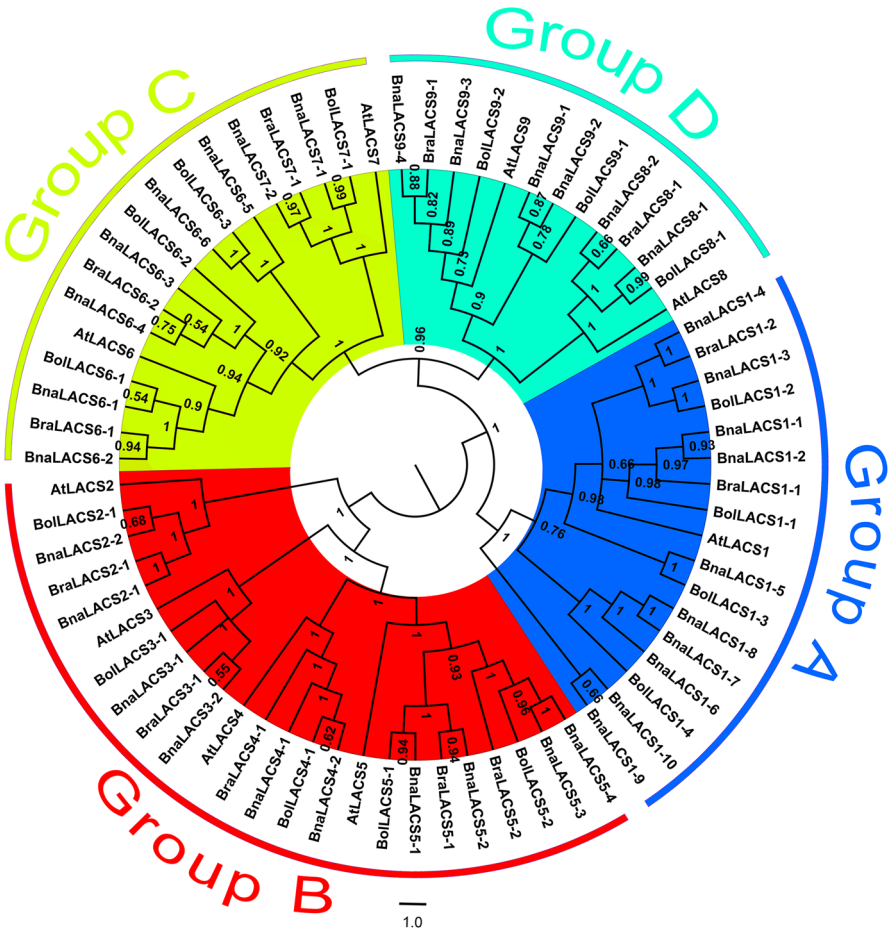

Fig. 1 The phylogenetic tree of LACS proteins from *A. thaliana*, *B. rapa*, *B. oleracea,* and *B. napus*. LACSs proteins were used to construct the NJ tree with 1000 bootstraps based on the protein sequences in *A. thaliana*, *B. rapa*, *B. oleracea,* and *B. napus* using MEGA 7.0 program. The LACS proteins were clustered into four groups

important for LACS proteins, and the remaining 15 conserved motifs were not annotated in InterProScan yet.

Previous study indicated that tertiary structure and evolution have a weak but recognizable correlation (Choi et al. 2007). To further examine the tertiary structural features of LACS proteins, we predicted the tertiary structures of LACS proteins in *A. thaliana* and *B. napus* using the online SWISS-MODEL workspace. As shown in Fig. S2, the tertiary structures of BnaLACS proteins in Group A and B are quite different, which implied a functional divergence of BnaLACSs. However, the tertiary structures of BnaLACSs in Group C and Group D are relatively conservative.

Expression Profiles of *BnaLACSs* in Various Tissues

To explore the roles of *BnaLACS*s, the expression patterns of all 34 *BnaLACS*s in various tissues of *B. napus* ZS11 at different developmental stages were investigated using RNA-seq dataset (BioProject ID PRJNA358784) (Zhou et al. 2017; Di et al. 2018).

The current study showed that the expression levels of most *BnaLACS* members differed in various tissues, suggesting the different functions of these genes (Fig. 3 and Table S3). Fourteen *BnaLACS* genes (*BnaLACS1-2*, *BnaLACS1-3*, *BnaLACS1-9*, *BnaLACS1-10*, *BnaLACS2*, *BnaLACS4*, *BnaLACS6-1*, *BnaLACS6-2*, *BnaLACS8*, *BnaLACS9-3,* and *BnaLACS9-4*) were highly expressed in nearly all tissues, which suggested that these genes play a sustainable role across *B. napus* growth. Conversely, eight *BnaLACS* genes (*BnaLACS1-1*, *BnaLACS1-6*, *BnaLACS1-7*, *BnaLACS1-8*, *BnaLACS6-5*, *BnaLACS6-6*, *BnaLACS9-1*, and *BnaLACS9-2*) were nearly not expressed in all tissues (Fig. 3 and Table S3). The expression abundances of the same gene in the same organs may be varied at different developmental stages, suggesting differences in the temporal expression. Besides, several genes (*BnaLACS5-1*, *5–2*, *5–3*, and *5–4*) are expressed only in buds, anthers, and stamens, indicating they may be expressed at specific organs. In short, a few *BnaLACS* genes have a relatively wider range of expression profiles while most of the genes are highly expressed only in individual tissues (Fig. 3 and Table S3).

To ensure the reliability of the expression patterns of *BnaLACS*s based on transcriptomic analysis, we selected 18 *BnaLACSs* with relative higher expression levels from different groups to analyze their expressions in 16 tissues or organs of ZS11 by qRT-PCR. Our result showed that the expression patterns from qRT-PCR were very similar to that of RNA-seq, which confirmed that the reliability and reproducibility of our study (Figs. 3, 4).

To further understand the roles of *BnaLACS*s for *B. napus* seed oil accumulation at seed developmental stage, we further analyzed the expressions of the 34 *BnaLACS*s at different seed developmental stages (weeks after pollination, WAP), based on previously published RNA-seq data (accession number GSE77637) from GEO in NCBI (https://www.ncbi.nlm.nih.gov/geo) (Wan et al. 2017). We found that eight *BnaLACS* genes (*BnaLACS4-1, 4–2, 6–1, 6–2, 8–1, 8–2, 9–3*, and *9–4*) were expressed in developmental seeds at the four stages, and six genes (*BnaLACS1-2, 1–3, 1–9, 1–10, 2–1*, and *2–2*) showed relatively higher expressions in seeds at 2

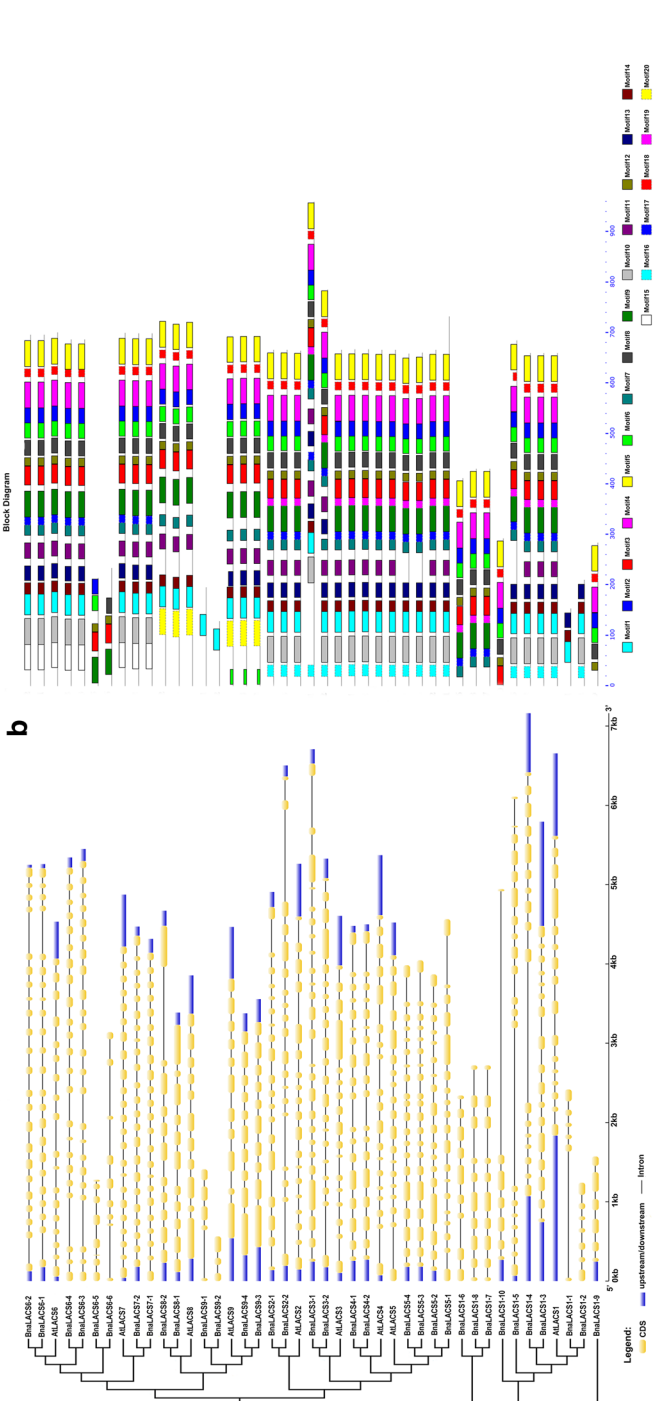

Fig. 2 **a** The exon–intron structure of the *AtLACSs* and *BnaLACSs* according to their phylogenetic relationships. An unrooted phylogenetic tree was constructed with the neighbor-joining method (1000 bootstrap replicates) based on the full-length protein sequences of 9 AtLACSs and 34 BnaLACSs. Exon–intron structure analyses of the *AtLACSs* and *BnaLACSs* were carried out using the online tool GSDS. The length of the exons and introns of each *AtLACS* and *BnaLACS* gene is proportional. **b** The conserved motifs of the LACS proteins in *A. thaliana* and *B. napus* according to their phylogenetic relationships. The conserved motifs were identified by MEME program. Each motif is indicated by a colored box numbered at the bottom and gray lines represent the non-conserved sequences. The length of motifs in each protein is proportional. At: *A. thaliana*; Bna: *B. napus*

WAP, 4 WAP, and 6 WAP stages. These results suggested the important roles of these genes in seed oil formation in *B. napus*. *BnaLACS7-1* and *7–2* have high transcriptional levels only in seeds at 8 WAP stage, which implied that both genes may play important roles in fatty acid oxidation (Fig. 5).

Comparative Expression Analysis of *BnaLACSs* in Two *B. napus* Cultivars with Different Oil Contents

In *Arabidopsis*, LACSs can activate free fatty acids into acyl-CoA thioesters, and thus they play a key role in fatty acid metabolism (Shockey et al. 2002). To further explore the roles of *BnaLACSs* in seed oil accumulation in *B. napus*, 19 *BnaLACS*s with relative high expression levels in seeds were further selected to compare their expression patterns in seeds at different developmental stages in two rapeseed cultivars with extremely high-(P1-HO, approximately 43% oil contents in seeds) or low oil content (P2-LO, approximately 33% oil contents in seeds) by qRT-PCR method.

As shown in Fig. 6, the expression levels of *BnaLACS1-10*, *4–1*, *6–4*, *9–3*, and *9–4* in P1-HO (high oil content) were significantly higher than that of P2-LO (low oil content) in almost all seed tissues investigated. This indicates that these five *BnaLACS*s may play a vital role in seed oil biosynthesis.

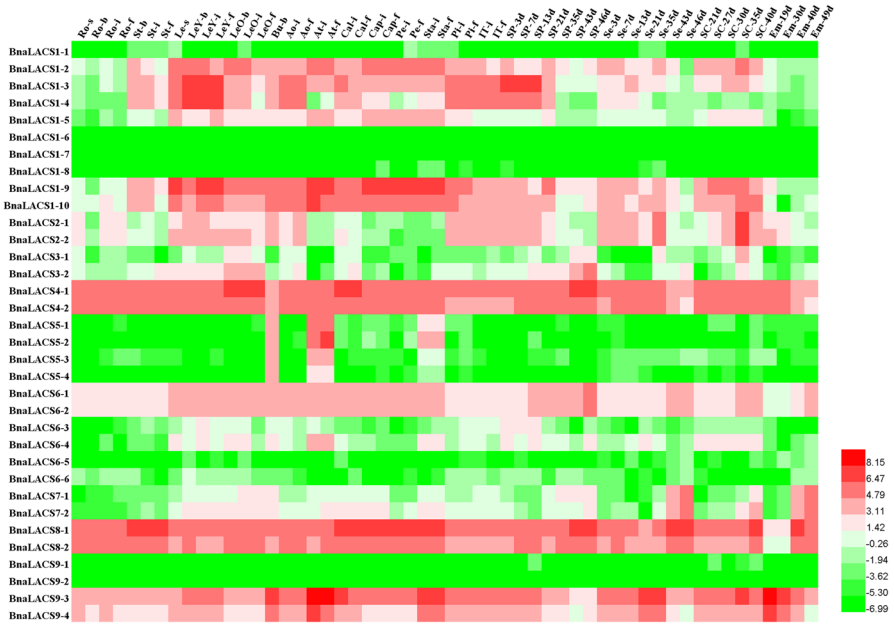

Fig. 3 The expression patterns of *BnaLACSs* in different tissues or organs at various developmental stages in *B. napus*. The abbreviations combinations above the heatmap indicate the different tissues or organs at different developmental stages from *B. napus* ZS11 (listed in Table S5). The heatmap was drawn using Heatmap Illustrator (HemI) (Deng et al. 2014). The bar on the lower right corner represents $\log_2$ expression values, and green represents low expression and red stands for high expression

Subcellular localization prediction showed that BnaLACS6-4, 9–3 and 9–4 proteins are located at the chloroplast, which is the site of de novo fatty acid synthesis in plants (Schnurr et al. 2002); BnaLACS1-10 and 4–1 are located at the nucleus and cytoplasm, respectively. Therefore, it was suggested that BnaLACS6-4, 9–3, and 9–4 may be involved in fatty acid synthesis in chloroplast, and BnaLACS1-10 and 4–1 may play a vital role in eukaryotic pathway of lipid biosynthesis. The comparative analysis and the subcellular localization prediction result revealed that BnaLACS6-4, BnaLACS9-3, and BnaLACS9-4 are the three main homologues involved in fatty acid synthesis and further seed oil content accumulation in *B. napus*.

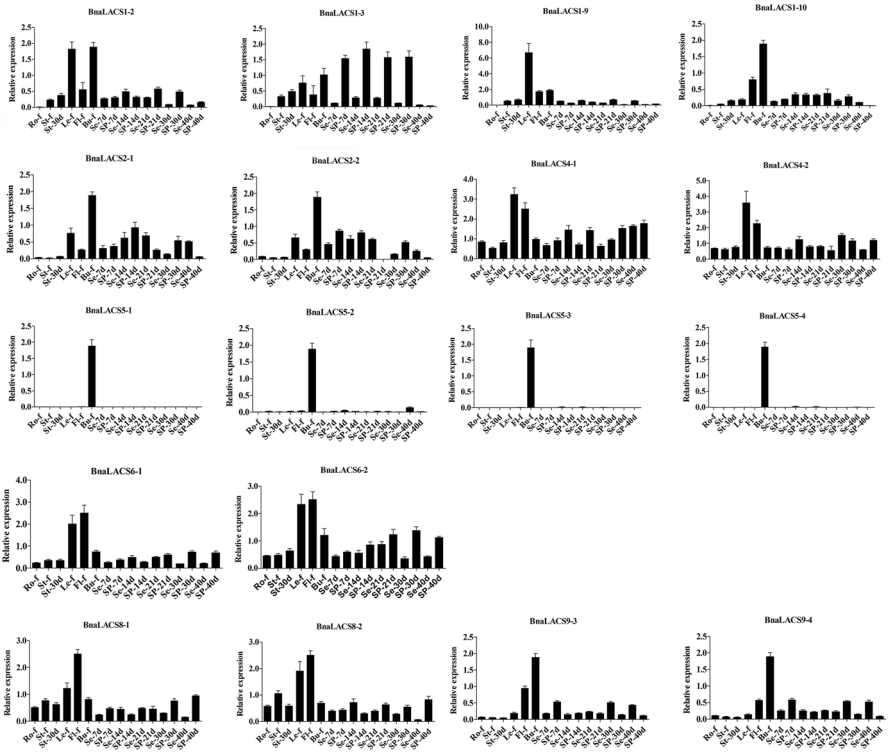

Fig. 4 Tissue expression profiles of selected 18 *BnaLACSs* at different developmental stages as determined by quantitative real-time PCR (qRT-PCR). The x axis corresponds to the different tissues and organs/developmental stages of *B. napus* "ZS11", roots (Ro-f); stems (St-f); leaves (Le-f); flower (Fl-f); bud (Bu-f) in the flowering period; seeds (Se-7d, Se-14d, Se-21d, Se-30d, Se-40d); silique pericarps (SP-7d, SP-14d, SP-21d, SP-30d, SP-40d) after flowering 7, 14, 21, 30, and 40 days, respectively. BnACTIN7 (EV116054) were used as the reference gene (Wu et al. 2010). Values represent the average ± standard error (SE) of three biological replicates with three technical replicates at each developmental stage. Error bars denote SE among three experiments

Discussion

To date, various functional research programs of *LACS* genes were conducted in many plant species, which proved the critical roles of long-chain acyl-coenzyme A (CoA) synthetases (LACSs) in fatty acid metabolism (Shockey et al. 2002; He et al. 2007; Wang and Li 2009; Tan et al. 2014). However, few related works have been applied in *B. napus*, a widely cultivated oil crop. In this study, we conducted a comprehensive analysis of the *LACS* gene family in *B. napus* genome, accompanied by expression assay of the candidate *BnaLACSs* in various tissues at different stages and between two extremely varieties with different oil contents.

In this study, a total of 12, 16, and 34 *LACS* genes were identified in the genomes of *B. rapa*, *B. oleracea*, and *B. napus*, respectively. The sum of *BraLACSs* and

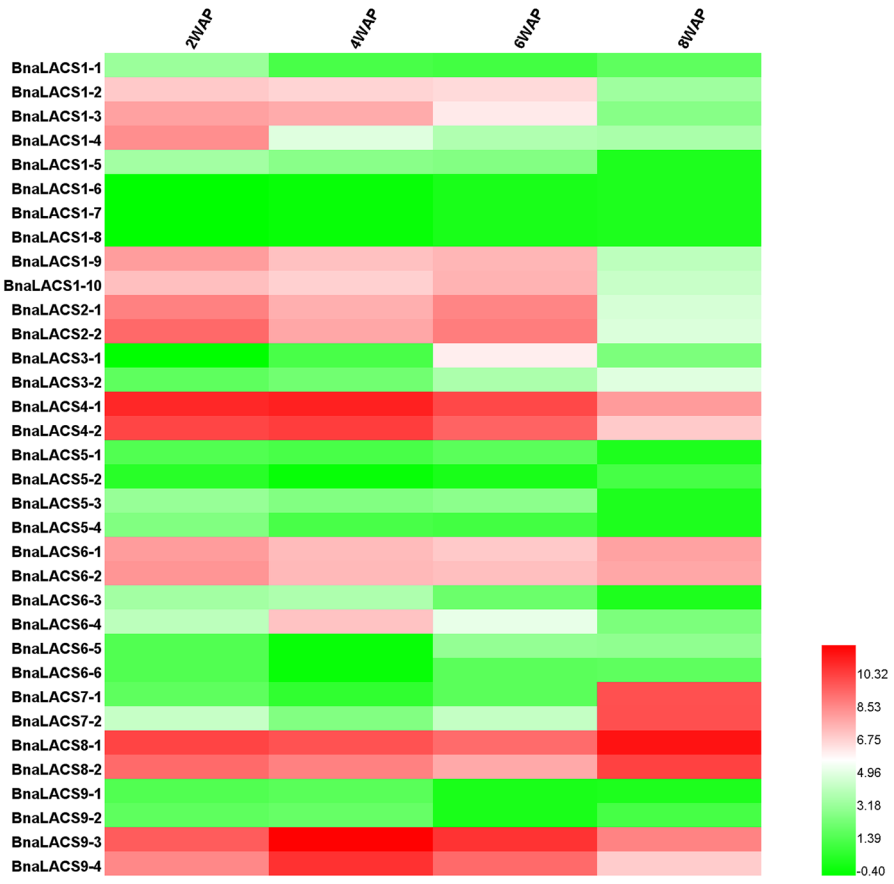

Fig. 5 Spatial-specific expression profiles of *BnaLACSs* in the process of seed development. The spatial-specific expression levels of 34 *BnaLACSs* were obtained from the RNA-seq data (accession number GSE77637). This heat map was generated based on the $\log_2$ expression values of 34 *BnaLACS* genes at seed developmental stage (weeks after pollination: WAP): 2 WAP, 4 WAP, 6 WAP, 8 WAP in *B. napus*. Green indicates low expression and red indicates high expression

BolLACSs is less than the number of *BnaLACSs,* which hinted that there may be gene redundancies in *BnaLACSs*. Molecular characterizations showed great variations among the 34 *BnaLACSs*, where the molecular weights and pIs of them were obviously different among groups; however, members in the same group possessed relatively similar molecular masses and pIs. It was shown that divergences in exon–intron structures can lead to changes of gene function and thus played a crucial role in the evolution of gene families (Xu et al. 2012). In our study, variant exon–intron structures of the *BnaLACS* genes in different groups revealed the diversification in gene structure. Moreover, we found that *BnaLACSs* with similar gene structure also have similar tertiary structures and conserved motifs. Notably, the gene structure, tertiary structures, and conserved motifs of *BnaLACS1-1*, *6–5*, *6–6*, *9–1,* and *9–2* are markedly different from those of the remaining *BnaLACSs*; consistent with this trend, these genes are hardly expressed in all tissues examined. Given some gene fragments in these genes were lost, it was suggested these fragments deletion may lead to loss of gene function.

Expression analyses have revealed that *LACS* genes have different expression patterns in various tissues in *Arabidopsis*, rice, cotton, and soybean (Fulda et al. 2002; Shockey et al. 2002; Ichihara et al. 2003; Wang and Li 2009). In *Arabidopsis*, most *LACS* genes have quite different expression levels in developing seeds; *LACS1*, *LACS2*, *LACS4*, and *LACS9* showed relative higher expression levels compared with *LACS6*, *LACS7,* and *LACS8*, and *LACS3* and *LACS5* are not expressed in the developing seeds (Shockey et al. 2002). In the current study, *BnaLACSs* also exhibited differential expression patterns in different tissues. Notably, *BnaLACS4-1*, *4–2*, *6–1*, *6–2*, *8–1*, *8–2*, *9–3*, and *9–4* were highly expressed in all tissues (Fig. 3), which is similar with their counterparts in *Arabidopsis* (Shockey et al. 2002). Similarly, some *BnaLACSs*, such as *5–1, 5–2, 5–3,* and *5–4,* were specifically expressed in buds, anthers, and stamens; accordingly, their homologous gene *AtLACS5* is specifically expressed in flowers (Shockey et al. 2002). And it was demonstrated that the *AtLACS4* homologous gene in *B. napus*, *BnaLACS4*, resided at endoplasmic reticulum (ER) and was involved in lipid biosynthesis as well (Tan et al. 2014). These results suggested that the functions of the *LACS* genes in organs of different species are somewhat conserved. Consequently, we speculated that these genes may have important functions in the process of lipid metabolism, just like the homolog genes in *Arabidopsis* (Zhao et al. 2010).

On the basis of the above RNA-Seq analyses, we intuitively observed that *BnaLACS1* (*1–2*, *1–3*, *1–4*, *1–9*, *1–10*), *BnaLACS2* (*2–1*, *2–2*), *BnaLACS4* (*4–1*, *4–2*), *BnaLACS6* (*6–1*, *6–2*, *6–4*), *BnaLACS7* (*7–1*, *7–2*), *BnaLACS8* (*8–1*, *8–2*), and *BnaLACS9* (*9–3*, *9–4*) exhibited higher expression levels during seed development. Subsequently, we further analyzed these 18 *BnaLACSs* to compare their expressions in seeds between high oil content (P1-HO) and low oil content (P2-LO) *B. napus* cultivars. The results showed that the expression levels of *BnaLACS1-10*, *4–1*, *6–4*, *9–3*, and *9–4* in P1-HO were significantly higher than that of P2-LO, which indicated that these genes may play a vital role in seed oil biosynthesis and high oil content formation in *B. napus*. Interestingly, we found two *AtLACS4* homologs, *BnaLACS4-1* and *4–2*, were highly conserved with 99.7% amino acid similarity (only have two different amino acids), but their expression patterns are quite different between

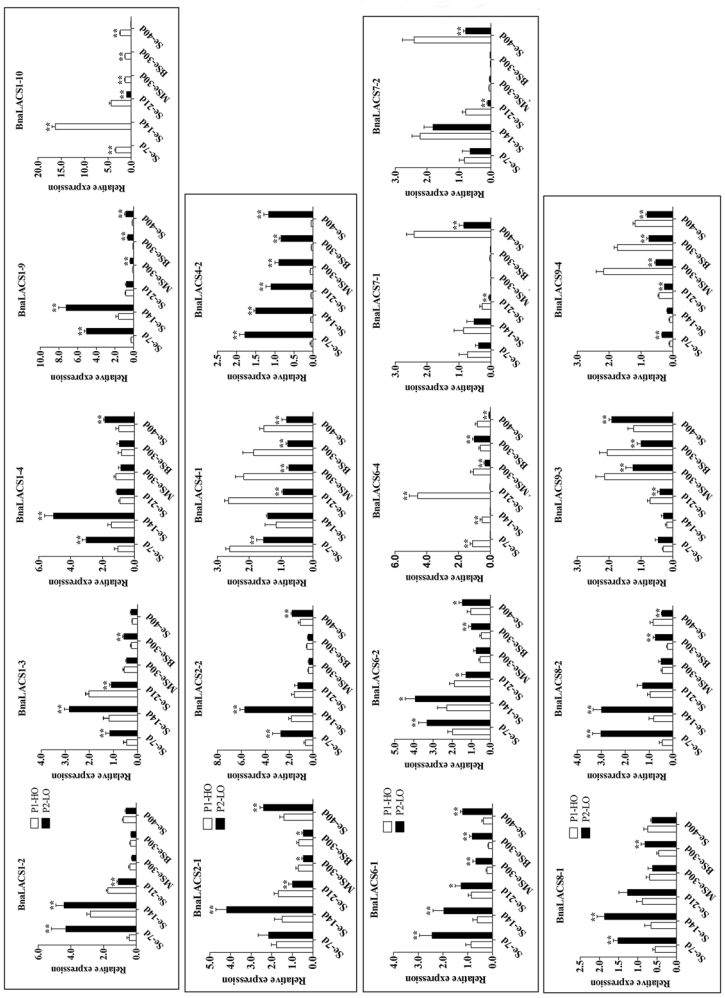

Fig. 6 qRT-PCR results of 18 selected *BnaLACSs* in seeds at different developmental stages between P1-HO (high oil content) and P2-LO (low oil content) Brassica napus. Seeds on the main inflorescence after pollination at 7 (Se-7d), 14 (Se-14d), 21(Se-21d), 30 (MSe-30d), 40 (Se-40d) days and seeds on the primary branches at 30 (BSe-30d) days were used for qRT-PCR analysis. Expression levels of BnaLACSs in P1-HO significantly different to P2-LO are indicated by asterisks (Student's *t* test, *: $p < 0.05$, **: $p < 0.01$)

high (P1-HO) and low oil (P2-LO) (Fig. 5). It was verified that *BnLACS4* (*BnaLACS4-2* in this study) was highly homologous to *AtLACS4* and could also complement a LACS-deficient yeast strain YB525 and even can increase the lipid content in the yeast strain pep4 by overexpression *BnLACS4*(Tan et al. 2014). The present study showed that the expression level of *BnaLACS4-1* in P1-HO was significantly higher than that of P2-LO, as compared to *BnaLACS4-2*. Moreover, *BnaLACS4-1* were successfully located within the QTL confidence interval associated with oil content (Chao et al. 2017). Based on these results, though the expressions of *BnaLACS4-1* and *4–2* were different that might due to the expression diversification occurred in the promoter regions of them, it was speculated that the *BnaLACS4s* homolog genes have important role in *B. napus* lipid biosynthesis as well. And *BnaLACS4-1* may play a relatively more important role in lipid biosynthesis and contribute to the formation of high oil content. Besides, the expression level of *BnaLACS1-10*, *6–4*, *9–3,* and *9–4* in P1-HO was also significantly higher than that of P2-LO, indicating their important roles in seed fatty acids biosynthesis and high oil content formation in *B. napus*. Taken together, the present study characterized the difference expressions of *BnaLACSs* during seed oil accumulation process, based on a comprehensive survey of phylogenetic relationships, gene structures, conserved motifs, and expression profiles of the *LACS* genes in different tissues of *B. napus*. And the comparative analysis of *BnaLACSs* in two *B. napus* cultivars with different seed oil contents revealed that *BnaLACS1-10*, *4–1*, *6–4*, *9–3*, and *9–4* genes may play important roles for long-chain fatty acid synthesis and further seed oil accumulation in *B. napus*. In the next study, we will further research the molecular functions of *BnaLACS1-10*, *4–1*, *6–4*, *9–3*, and *9–4* genes and explore the application potential of these genes in improving the seed oil content of *B. napus*. In general, the current study provides a foundation in further understanding the characterization of *LACS* genes family and for molecular breeding studies to increase the seed oil content and quality in *B. napus*.

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Author Contributions J.N.L. and N.N.L. conceived and designed the experiments. Z.C.X. performed the experiments. Z.C.X., S. F.W., J.J.S., L.Y. Z., C.Z., H.Y., H.Y.Z., B.Y., L.J.W., H.D., C.M.Q, and K.L. analyzed the data. Z.C.X. and N.N.L. wrote the paper. All authors reviewed the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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