Genome-Wide Identification and Expression Profiling Analysis of The Long-Chain Acyl-CoA Synthetases Reveal Their Potential Roles in Wheat Male Fertility

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Abstract: Long-chain acyl-CoA synthetase (LACS), responsible for the conversion of free FAs into acyl-CoAs, is involved in multiple pathways of lipid metabolism. Although LACS genes in Arabidopsis have been well characterized, no detailed information concerning this family is available for wheat. In the present study, a systematic analysis was carried out for the wheat LACS family. As a result, 30 putative TaLACSs were identified. Expression analysis revealed that 22 TaLacs were expressed in wheat anthers. Two orthologs of AtLACS1, TaLACS2 and TaLACS3, were repressed at the vacuolated stage in the cold-treated BS366 (a temperature-sensitive genic male-sterile line). Thus, TaLACS2 and TaLACS3 may function like AtLACS1 in wax biosynthesis in anthers, and the repression of both genes may be correlated with the male sterility of BS366. TaLACS5 is an ortholog of AtLACS5, which was expressed exclusively in anthers. TaLACS5 was repressed in the cold-treated BS366 at the tetrad, uninucleate, and vacuolated stages. The negative correlation between TaLACS5 and TaGAMYB-B, and the MYB domain found in the promoter sequence suggested that TaLACS5 may be negatively regulated by TaGAMYB-B to participate in wheat fertility. These findings will provide a valuable foundation for the understanding of the wheat LACS gene family in male fertility.

Keywords: long-chain acyl-CoA synthetase; anther; fatty acids; male sterility; cuticular wax

1. Introduction

Fatty acids (FAs) are virtually ubiquitous in plant cells, and serve as building blocks for a variety of lipids. FAs are incorporated into membrane glycerolipids, sphingolipids and storage triacylglycerols, where they serve as precursors of surface waxes, cutin and suberin [1]. Previous studies have reported their roles in membrane integrity, energy supply and responses to abiotic and biotic stresses [2–4]. Long-chain acyl-CoA synthetase (LACS), responsible for the conversion of free FAs into acyl-CoAs, is involved in multiple pathways of lipid metabolism, such as fatty acid transport, lipid synthesis, and fatty acid β-oxidation [5,6].

Arabidopsis contains one of the largest known LACS families, with nine LACS genes, most of which have been well characterized [1,6]. Characterization of LACS mutants and analysis of expression patterns and subcellular localizations has disclosed a complex network of redundant or moderately redundant LACS activities involved in different aspects of lipid metabolism in Arabidopsis [1]. AtLACS1, AtLACS2 and AtLACS4 were reported to localize in the endoplasmic reticulum (ER) and participated in the activating of fatty acids for the production of cuticular lipids. AtLACS1 has a primary role in generating very-long-chain fatty acyl-CoAs that serve as precursors for cuticular wax components. AtLACS2 seems to function by overlapping with AtLACS1 in the activation of very long-
chain fatty acids (VLCFAs) in the wax component production, and plays a role in the incorporation of C16 and C18 acyl groups into cutin [7–9]. MdLACS2, from apple, an ortholog of AtLACS2, was shown to catalyze the formation of 16:0 CoA [10]. BnLACS2 from rapeseed involved in seed oil production, exhibited a substrate preference for 14:0, 16:0, 18:0, 18:1, and 22:1 Co [11]. The ER-resident AtLACS4 was also reported to be partially redundant with AtLACS1 in providing a substrate for cuticular wax biosynthesis [9]. AtLACS4 and AtLACS8 are both ER-localized [12]. AtLACS9 is the only LACS gene exclusively localized in the outer membrane of the plastid envelope [13]. Both AtLACS4 and AtLACS9 are involved in the lipid trafficking between the ER and plastid for glycerolipid synthesis [12]. AtLACS8 functionally overlaps with AtLACS4 and AtLACS9, as the disruption of AtLACS8 in the lacs4lacs9 double mutant results in lethality [12]. In contrast to the above-mentioned LACS members, both AtLACS6 and AtLACS7 located in peroxisome have an overlapping role in fatty acid β-oxidation [14,15]. Although no abnormal phenotype was observed for the single mutant of AtLACS6 or AtLACS7, the lacs6lacs7 double mutant was defective in seed oil mobilization [15]. The function of AtLACS3 and AtLACS5 is still unclear. It has been suggested that AtLACS3 is only expressed in the root, stem, leaf, and flower, whereas AtLACS5 is exclusively detected in anthers [6,16]. The expression pattern of LACS genes has also been extensively investigated in other plant species. In B. napus, 18 of 34 BnLACSs expressed in developing seeds [17], which implicated their major roles in lipid metabolism. The expression of BnLACS4s, BnLACS8s, and BnLACS9 was quite similar to their closest Arabidopsis orthologs [17]. MdLACS in apple are highly expressed in pericarp tissues where wax and cutin are actively produced, which suggests their possible roles in cuticle synthesis [18]. Several BnLACS5 genes are specifically expressed in buds, anthers, and stamens, which are similar to AtLACS5 [17]. One cotton ortholog of AtLACS5, GhACS1, is predominantly accumulated in the anther and plays an essential role in microsporogenesis in the anther development of cotton [19]. Accordingly, it will be intriguing to investigate its role in wheat fertility.

The utilization of heterosis is an important approach to increasing wheat yield, to ensure food security. Known as the prerequisite component of the hybrid system, the male-sterile line directly determines the hybrid yield and seed purity. Thus, a study of the mechanisms underlying male sterility will undoubtedly facilitate its utilization in hybrid breeding [20]. Although the function of LACS and their potential roles in male sterility have been reported in other plants, the role of LACS in wheat still remains unclear. Therefore, a genome-wide analysis of LACS genes will be of great value to understanding the function of the LACS gene family in wheat fertility. In this study, a total of 30 LACS genes were identified through a genome-wide gene family search. Subsequently, the gene phylogenetic relationship, gene structure, protein-conserved domain, chromosome localization, cis-acting regulatory elements and expression profiles were systematically analyzed to specify the evolutionary and functional features of TaLACSs. In addition, the relative expression of several LACS genes was determined in a temperature-sensitive genic male-sterile (TGMS) line, Beijing Sterility 366 (BS366), to better understand their function in anther and pollen development [21]. This work could contribute to a better understanding of LACS genes in wheat fertility.

2. Results
2.1. Identification and Annotation of LACS Gene Family Members in Wheat

Nine AtLACS protein sequences were used as the query sequence for BLASTP against the wheat genome with an e-value of 1e-20. The hidden Markov model (HMM) file for AMP-binding domain (PF00501) was aligned with all the amino acid sequences in the wheat. The obtained AMP-binding genes were filtered, using the Pfam, SMART (http://smart.embl.de/ (accessed on 8 April 2022)), and CDD (https://www.ncbi.nlm.nih.gov/cdd/ (accessed on 10 April 2022)) in NCBI. Eventually, 30 putative LACS family genes were identified from both methods. TaLACSs were unevenly
distributed on wheat chromosomes, with one–five genes located on each chromosome (Supplementary Table S1), and the number of TaLACSs in the A, B, and D genome were the same (10 genes in each sub-genome). The 30 putative TaLACSs were renamed TaLACS1 to TaLACS30, based on their chromosomal locations (Supplementary Table S1), and the length of peptides encoded by these TaLACSs ranged from 475 amino acids (aa) to 728 amino acids (aa). The theoretical molecular weights of the wheat LACS proteins varied from 52.48 to 79.93 kDa. The theoretical pI values ranged from 5.54 to 8.57 (Supplementary Table S1).

2.2. Phylogenetic and Gene Structure Analysis of The LACS Gene Family Members

To detect the evolutionary relationships of LACS proteins among wheat and Arabidopsis, an un-rooted neighbor joining (NJ) tree was constructed using the full-length amino acid sequences of nine Arabidopsis and 30 wheat LACS proteins (Figure 1). The phylogenetic analysis revealed that the LACS could be naturally grouped into four major clades, named Clade I to IV. These clades contained 9, 6, 6, and 9 wheat LACS proteins, respectively. There are 2, 2, 2, and 3 AtLACS in clade I to IV, respectively. All of the wheat LACS clustered together with three highly homologous genes.

![Phylogenetic tree of the wheat and Arabidopsis LACS family.](image)

**Figure 1.** Phylogenetic tree of the wheat and Arabidopsis LACS family. An un-rooted neighbor joining (NJ) tree for LACS proteins was constructed, based on the amino acid sequence alignments of wheat and Arabidopsis LACS. All the proteins are clustered into four clades shown in different circles with different colors.

2.3. Motif Composition and Gene Structure of The TaLACS Genes
To further understand the evolutionary characteristics of the LACS gene family in wheat, the protein motif and gene structure were analyzed in this study. A phylogenetic tree was constructed, using the full amino acid of wheat LACS proteins. The conserved motifs of 30 wheat LACS proteins were analyzed, using the MEME Suite tool. This analysis generated 15 putative conserved motifs for all wheat LACS proteins. As shown in Figure 2, motifs of all the wheat LACS proteins were classed into four clades. There are 15 conserved motifs for proteins in clade III (green) and IV (blue). All six proteins in clade II (purple) have 15 motifs except TaLACS9 and TaLACS10. Motif 12 was not found in TaLACS10. Motifs 4, 9, 10, 12, and 13 were not found in TaLACS9. Most proteins in clade I (red) have 15 motifs except TaLACS16, TaLACS21, and TaLACS26. Motif 15 was not found in these proteins. The exon/intron structures of LACS genes were further analyzed and presented (Figure 2b). The exon/intron structures in the TaLACSs varied among different clusters, but were relatively conserved within the same cluster. All of the TaLACSs in clade I contain 10 exons. TaLACSs in clade II contain 16–23 exons, with 16 exons in TaLACS19, and 21 exons in TaLACS10. There are 23 exons in TaLACS7, TaLACS10, TaLACS14, and TaLACS24 proteins. All the proteins in clade III contain 19 exons. All of the proteins in clade IV contain 19 exons except TaLACS28, which includes 18 exons.

Figure 2. Phylogenetic relationship, conserved motif, and gene structure analysis of TaLACS genes. (a) Phylogenetic tree of wheat LACS proteins. (b) Conserved motifs of wheat LACS proteins. (c). Exon-intron structures of TaLACS genes. Fifteen conserved motifs are shown in different colored boxes, as indicated on the right of the figure. Green boxes in (c) represent exons, black lines represent introns, and the upstream/downstream regions of TaLACS genes are represented by red boxes.

2.4. Chromosomal Location and Gene Synteny Analysis

In line with the available wheat genome annotation information, a total of 30 LACS genes were mapped onto 15/21 chromosomes. All the TaLACSs were present in triads. LACS genes were evenly distributed on the wheat chromosomes. There are ten TaLACSs located in the A, B, and D sub-genome, respectively. The number of TaLACSs on each chromosome was different. As shown in Figure 3, there are one, one, two, five, and one
TaLACS genes located on linkage one, three, four, five, and seven for the A, B, and D sub-genome, respectively. More than one TaLACS was found on chromosome 4A, 4B, and 4D, and 5A, 5B, and 5D (Figure 3). Tandem duplications were characterized as multiple members of one family occurring within the same intergenic region or in neighboring intergenic regions [22]. According to previous research, a tandem duplication event is defined when there are two or more genes inside 200 kb [23]. As a result, two genes in linkage four and five genes in linkage five were not identified as tandem repeats. These results indicate that tandem duplications might not contribute to the expansion of this gene family.

Figure 3. Synteny relationships of LACS genes in wheat. The outer circle segment represents the wheat chromosomes including “unchromosomes”. Un denotes genes with unknown physical positions. Link lines in the circle represents segmental duplication pairs between TaLACSs. The blue and red lines represent the segmental duplication pairs between the homologous and paralogous TaLACSs.

2.5. Cis-Acting Elements in The Promoters and GO Annotations of TaLACSs

Cis-acting element prediction in the promoters and Gene Ontology (GO) annotation will be of much help in the understanding of the TaLACS functions in wheat. Transcription factors (TFs) represent an important group of regulators involved in the regulation of gene expression at the transcriptional level. Cis-acting elements in the promoter are crucial regions of the binding site of the transcription factors for initiating transcription and gene expression. To gain an insight into the potential regulatory mechanism of TFs in the expression of LACS, the 2000 bp upstream promoter regions of all TaLACSs were used to predict the cis-acting regulatory elements via PlantCARE. All the cis-acting elements in the promoters of TaLACSs were classified into three categories, including phytohormone, abiotic/biotic stress, and growth and development (Figure 4a). The average number of cis-
acting elements related to growth and development were the highest (54), followed by abiotic/biotic stress (27), and phytohormone (16). The top three genes with most cis-acting elements were TaLACS21, TaLACS19, and TaLACS23 in growth and development, TaLACS6, TaLACS25, and TaLACS16 in abiotic/biotic stress, and TaLACS16, TaLACS19, and TaLACS23 in phytohormone (Figure 3a). Cis-acting elements involved in growth and development, including CAAT-box, TATA-box, as-1, CCGTCC motif, A-box, and CCAAT-box were found in the promoter sequences of TaLACSs. As shown in Figure 4b, cis-acting elements including CAAT-box and TATA-box were found in the promoters of all the TaLACSs. CCAAT-box and as-1 were identified in the promoters of 22 and 20 TaLACSs, respectively. Cis-acting elements involved in abiotic/biotic stress, including GATA-motif, G-Box, STRE, DRE, WRE3, ARE, LTR, and MBS were also found in the promoter sequences of TaLACSs. In total, 29 TaLACSs contain GATA-motif and G-Box. 27 and 21 TaLACSs contain STRE and DRE, respectively. Cis-acting elements involved in the phytohormone responses were also found in the promoters of TaLACSs. MeJA-responsive elements CGTCA-motif and TGACG-motif were found in all the TaLACSs’ promoter sequences. Abscisic acid responsive element, ABRE, was found in the promoter sequences of 29 wheat LACS genes. The TGA-element involved in auxin responsiveness was found in the promoter sequences of 15 genes (Figure 4b). MYB and MYC elements were found in all the promoter sequences of TaLACSs, which may suggest that the expression of TaLACSs may be regulated by MYB/MYC to participate in corresponding biological processes (Supplementary Table S2).

Figure 4. Cis-acting elements in the promoters of wheat TaLACS genes. (a) Number of elements assigned to biotic/abiotic stress, growth and development, and phytohormone responses for each TaLACS. (b) Cis-acting elements in the promoters of TaLACS genes in wheat. The different shades of red represent the number of cis-acting elements.

In this study, GO annotation was carried out for all the TaLACSs. There are 29, 27, and 7 genes assigned to the biological process, molecular function, and cellular component, respectively. TaLACSs were annotated in biological processes including the cutin
biosynthetic process, the fatty acid metabolic process, the long-chain fatty acid metabolic process, the phenylpropanoid metabolic process, the defense response to fungus, and lateral root formation. The majority of TaLACSs (21/30) were assigned to the fatty acid metabolic process. TaLACSs were predicted to function in cellular components such as the endoplasmic reticulum, plasmodesma, membrane, and integral components of the membrane. TaLACSs were predicted to have 4-coumarate-CoA ligase activity, trans-cinnamate-CoA ligase activity, long- or very-long-chain fatty acid-CoA ligase activity, ATP-binding catalytic activity, and nucleotide-binding activity (Supplementary Table S3).

2.6. The Expression of TaLACS Genes in Wheat

To gain a general understanding of the TaLACS expression in different tissues, the expression data from five wheat tissues (root, stem, leaf, spike, grain) of Chinese Spring were used in this study. In total, 29 genes were found to express in at least one tissue (FPKM > 1). The number of the expressed TaLACSs ranged from 22 (leaf) to 28 (stem) in different tissues. There was only one tissue-specific gene among all the TaLACS genes. TaLACS25 expressed only in root tissue. TaLACS28, TaLACS29, and TaLACS30 expressed in both root and stem tissues. TaLACS11 expressed in grain, spike, and stem tissues. TaLACS17, TaLACS27, and TaLACS22 expressed in grain, leaf, spike, and stem tissues. TaLACS7 and TaLACS9 expressed in grain, root, spike, and stem tissues. The remaining 19 genes expressed in all five tissues. Based on the expression patterns from five tissues in Chinese Spring, TaLACS genes can be classified into two clusters (Figure 5a). Genes in cluster I expressed highly in root, stem, and leaf tissues. Genes in class II showed high expression levels in the spike and grain tissues. Genes in clusters I and II can be further classified into two subclusters. Genes in subcluster I-1 are highly expressed in the root, stem, and leaf tissues, while genes in subcluster I-2 were abundantly expressed in the root and stem tissues. Genes in subcluster II-1 were most abundant in the grain tissue. Genes in subcluster II-2 were more abundant in the spike and grain tissues compared with the other three tissues. All of these results suggest that TaLACSs might participate in many aspects of biological functions in different tissues, as they express in all five plant tissues.

To further explore the potential roles of TaLACS in anther and pollen development, the expression of all the TaLACSs was examined in the transcriptome sequencing data of anthers from the early uninucleate, vacuolated, binucleate, and trinucleate pollen stages. Heatmaps of TaLACS genes were generated using the expressed genes (average FPKM > 1). As shown in Figure 5b, the expression of expressed TaLACS genes can be clustered into three clusters. Genes in cluster I express highly at the uninucleate stage. Genes in cluster II are mainly expressed at the middle two stages, with higher expression at the vacuolated stage and moderate expression at the binucleate stage. Genes in class III are highly expressed at the last stage. These results indicate that TaLACS may be important for four respective development stages in anther or pollen development. Among 30 TaLACSs, the average expression of 2 TaLACS genes was lower than 1 FPKM; the expression of nine genes ranged from one to ten FPKM; the expression level of 17 genes ranged from 10 to 100 FPKM; the expression of two genes was higher than 100 FPKM (Figure 5c). The number of genes expressed were 27, 28, 26, and 23 for the early uninucleate, vacuolated, binucleate, and mature pollen stages, respectively. A total of 22 genes were found expressed in four stages (Figure 5d). Only one gene, TaLACS28, was mature pollen-stage specific. TaLACS29 expressed at both the vacuolated and binucleate stages. TaLACS11 and TaLACS9 expressed at both the uninucleate and vacuolated stages. TaLACS17, TaLACS8, and TaLACS7 expressed at three early stages. Five genes were highly expressed in wheat anthers (FPKM > 100), with three genes (TaLACS17, TaLACS22, and TaLACS27) at the early uninucleate stage, and two genes (TaLACS1 and TaLACS3) at the middle two stages (Figure 5e). All these results indicate that TaLACS genes may participate in late anther development, during which the microspores develop into mature pollen grains.
Figure 5. Expression of TaLACSs in wheat. (a) Expression heatmap of TaLACSs in five wheat tissues. (b) Expression heatmap of TaLACSs genes in wheat anthers. (c) The expression range of TaLACSs in wheat anther at four stages. (d) Venn diagram of TaLACSs with expression level higher than 1 FPKM in wheat anthers. (e) The expression of the top five expressed TaLACSs in wheat anthers. UN, uninucleate stage; VA, vacuolated stage; BI, binucleate stage; TR, trinucleate stage.

2.7. Correlation between TaLACSs and Transcription Factors in Wheat Anther

To further study the role of TaLACS in wheat anther development, transcription factors reported to be involved in anther development were identified in the wheat anther transcriptome sequencing data. TaTGA9-B, TaTGA9-A, TaTGA9-D, TaTGA10-D, TaTGA10-B, and TaTGA10-A are orthologs of the AtTGA9 and AtTGA10, which encode a basic leucine-zipper (bZIP) transcription factor. It has been reported that AtTGA9 and AtTGA10 are redundantly required for anther development [24]. TaIG1-B is an ortholog of maize IG1, which encodes a LBD domain (LBD) transcription factor [25]. TaAMS-A is an ortholog of ABORTED MICROSPORE (AMS) in Arabidopsis [26]. TaTIP2-B and TaTIP2-D are orthologs of TIP2 in rice [27]. TaAMS-A, TaTIP2-B, and TaTIP2-D all encode the basic helix-loop-helix (bHLH) transcription factors. TaGAMYB-D, TaGAMYB-A, and TaGAMYB-B are orthologs of HvGAMYB in barley [28]. TaMYB65-A encodes a protein orthologous to MYB DOMAIN PROTEIN 65 (MYB65) in Arabidopsis [29]. The correlation between those 14 transcription factors and TaLACSs in the wheat anthers was analyzed (only |Pearson correlation coefficient| higher than 0.8 was considered here) (Figure 6a).

The correlations between TaLACSs and transcription factors were clustered into three sections. In the first cluster, TaLACSs were positively correlated with TaIG1-B, TaTIP2-B, TaTIP2-D, and TaAMS-A, and negatively correlated with TaTGA9-B, TaTGA9-A, TaTGA9-D, and TaGAMYB-B. The correlations in the second cluster were the opposite. TaLACSs in the second cluster were positively correlated with TaTGA9-B, TaTGA9-A, TaTGA9-D, and TaGAMYB-B, and negatively correlated with TaIG1-B, TaTIP2-B, TaTIP2-D, and TaAMS-
A. TaLACSs in the third cluster were negatively correlated with TaTGA10-A, TaTGA10-B, TaTGA10-D, and TaGAMYB-D. TaMYB65-A was positively correlated with only two genes (TaLACS16 and TaLACS21).

To further investigate the possible roles of TaLACS in anther development, quantitative RT-PCR (qRT-PCR) was used to measure the expression patterns of selected TaLACS and potential correlated transcription factors in the wheat anthers (Figure 6b). The expression of transcription factors including three TaTGA9, three TaGAMYB, and TaMYB65-A were examined in this study. The expression of transcription factors including TaMYB65-A, TaGAMYB-D, TaGAMYB-B, and three TaTGA9 increased gradually with the extension of time and decreased at the trinucleate stage. TaMYB65-A expressed at the highest level at the vacuolated and binucleate stages. TaGAMYB-A expressed at the highest level at the uninucleate and binucleate stages. The expression of TaGAMYB-D, TaGAMYB-B, and three TaTGA9 coding genes started to increase from the uninucleate stage, with the highest expression level at the binucleated stage. TaLACS21 and TaLACS26 up-regulated from the uninucleate stage to the binucleated stage. The similar expression pattern between TaLACSs and TFs, including three TaTGA9 and TaGAMYB-B, suggested their positive correlation roles in late anther and pollen development.

Figure 6. Expression correlation analysis among TaLACS genes and transcription factors in wheat anther. (a) Expression correlation analysis among TaLACS genes and transcription factors. Red indicates positive correlation; blue indicates negative correlation. Pearson correlation coefficients between genes were shown in the boxes of heatmap. (b) Expression analysis of selected transcription factors and TaLACS in wheat anthers.
2.8. Expression of TaLACS in The Temperature-Sensitive Genic Male-Sterile Line

Wheat temperature-sensitive genic male-sterile (TGMS) line BS366 (Beijing Sterility 366), is normal at 20 °C (control) but produces sterile pollen at 12 °C (cold) with 12 h of daylight. At the early uninucleate stage, free microspores are released from the tetrads. Microspores are spherical with thin exines (Figure 7a,e). Tapetal cells reabsorb their vacuoles, and the cytoplasm becomes condensed (Figure 7i,m). No differences were observed between the cold- and control-treated BS366. At the vacuolated stage, thicker exine are formed on the outer surface of the microspores. The microspore vacuolates with an increase of volume, resulting in a round-shaped microspore (Figure 7b). Tapetal cells become more degenerated, and the middle layer becomes invisible (Figure 7g). However, the pollen grains of cold-treated BS366 were irregularly spherical (Figure 7f,n). At the binucleate stage, the vacuolated microspore undergoes the first mitotic division with asymmetric cell division, generating a much smaller generative cell and a larger vegetative cell. As the starch accumulates inside the microspore, the vacuole diminishes gradually (Figure 7c). The tapetum cells almost completely degenerated (Figure 7k). In the cold-treated pollens, no starch accumulated in the pollen grain and the endothecium became thicker compared with the control-treated BS366 (Figure 7g,o). At the mature stage, the fertile pollen grains are full of starch (Figure 7d). The epidermis and the endothecium degenerate further, and the tapetum completely disappears (Figure 7i). Anther dehiscence occurs and mature pollen grains are released (Figure 7i). Pollen in the cold-treated BS366 were vacuolated and shrank (Figure 7h). The endothecium became abnormally expanded and thicker (Figure 7p).

Figure 7. The microspores and anthers at different developmental stages. Microspores at early uninucleate stage (a and e), vacuolated stage (b and f), binucleate stage (c and g), and mature pollen stage (d and h). Wheat anther at early uninucleate stage (i and m), vacuolated stage (g and n), binucleate stage (k and o), and mature pollen stage (i and p). (a–e) and (i–l), pollens and anthers of control-treated BS366; (e–h) and (m–p), pollens and anthers of cold-treated BS366; E, epidermis; En, endothecium; ML, middle layer; Msp, microspores; T, tapetum; bars in (a–h) 20 μm; bars in (i–p) 50 μm.
Pollen development after meiosis involves pollen exine development, starch accumulation, and nuclear mitosis. It has been reported that lipid metabolism plays an important role in pollen exine formation. To explore the roles of *TaLACS* in BS366 during pollen and late anther development, the expression of 11 *TaLACSs* and *TaTGA9-D* were studied in BS366 anthers under the cold and control conditions. As the pollen exine formation takes place before the early uninucleate stage, the expression of those genes was checked at the tetrad stage. As shown in Figure 8, *TaLACS1*, *TaLACS2*, and *TaLACS3* are three orthologs of *AtLACS1*. The expression of the three genes started to accumulate from the early uninucleate stage, and decreased at the binucleate stage. *TaLACS2* and *TaLACS3* were significantly repressed at the vacuolated stage, but all three *TaLACSs* were induced at the binucleate and trinucleate stages. *TaLACS5* was homologous to *AtLACS5*. It was differentially expressed at all five stages. The expression of *AtLACS5* was repressed under cold conditions at both the tetrad and vacuolated stages, but induced at the other three stages. *TaLACS8* and *TaLACS11* were two wheat homologues and clustered together with *AtLACS9*. Both genes were induced in the cold-treated BS366 at all stages except the vacuolated stage. *TaLACS16* was clustered together with *AtLACS8*. It was differentially regulated at both the tetrad and trinucleate stages. Although *TaLACS17*, *TaLACS22*, and *TaLACS27* were three wheat homologues, the expression patterns were different for the three genes. The expression of the three genes increased from the tetrad stage and peaked at the binucleate stage. All three genes were induced at the trinucleate stage. *TaLACS17* and *TaLACS27* were both induced by the cold treatment. Only *TaLACS17* was significantly induced at the tetrad stage. The expression of *TaLACS25* increased over time in the cold- and control-treated BS366, but the expression of *TaLACS25* was higher under the cold condition at the binucleate stage. The expression of transcription factors in Figure 6 were also examined in the cold- and control-treated BS366. The expression of *TaTGA9-A* and *TaTGA9-B* was similar. The expression of both genes was repressed at two later stages in the cold-treated BS366. However, the expression of *TaTGA9-D* peaked at the last two stages and was induced by the cold treatment. The expression of *TaTGA9-D* was repressed at the tetrad stage, but increased at the trinucleate stage. The expression of *TaMYB65-A* was significantly repressed at the vacuolated and binucleate stages. *TaGAMYB-A* was repressed at the tetrad stage. In addition, all three *TaGAMYB* transcription factors were repressed at the binucleate stage in BS366 under cold conditions. All the differential regulation of the LACS genes and transcription factors in BS366 between the cold and control conditions suggested their potential roles in the male sterility of BS366.
Figure 8. Expression of LACS genes and transcription factors in BS366 at different developmental stages between cold and control conditions. Asterisks indicate significant differences between cold and control conditions (Student's t-test, * p-value < 0.05, ** p-value < 0.01). TD, tetrad stage; UN, uninucleate stage; VA, vacuolated stage; BN, binucleate stage; TN, trinucleate stage.

3. Discussion

In flowering plants, the life cycle alternates between diploid sporophyte and haploid gametophyte generations. Male gametophytes in wheat develop from the initiation and generation of the male reproductive structure stamen, which consists of three anthers and filaments supporting each anther. Anthers are the male reproductive organs that generate pollen grains. The reproductive stage is directly related to seed production and life continuation. Understanding the molecular mechanism of anther and pollen development is crucial for future hybrid breeding [30]. During anther development, tapetum formation and apoptosis, meiosis, callose generation and degradation, and pollen wall formation, are closely related to pollen development [31]. Fatty acids and their derivatives are essential components of anther cuticle and pollen wall development. LACS responsible for the conversion of free fatty acids into acyl-CoAs, are involved in multiple pathways of lipid metabolism, such as fatty acid transport, lipid synthesis, and fatty acid β-oxidation [5,6].

To date, functional research on LACS genes has been conducted in many species, proving the critical roles of LACSs in fatty acid metabolism [6,32–34]; however, few related works have been applied to wheat. In this study, a genome-wide identification and expression analysis in the anther were carried out in common wheat. Finally, a total of 30
LACS genes were identified in the wheat genome. The identification of wheat LACS genes and their homologous relationships between AtLACS will be helpful in the understanding of their corresponding roles in wheat. Arabidopsis contains nine LACS genes, seven of which have been well characterized [1]. In this study, TaLACS1, TaLACS2, and TaLACS3 clustered together with AtLACS1, TaLACS17, and TaLACS22, and TaLACS27 with AtLACS2. It has been reported that AtLACS1 was involved in wax synthesis, and the mutation of AtLACS1 caused a strong deficient glossy phenotype [7]. AtLACS2 is mainly involved in cutin biosynthesis. The mutation of AtLACS2 caused strong cutin deficiency, but had little effect on wax biosynthesis under normal growth condition [35]; however, it has been reported that AtLACS2 is also involved in wax synthesis under stress conditions [36]. However, recent studies reported that AtLACS2 appears to function overlapping with AtLACS1 in wax and cutin production [9,14].

The anther cuticle is an extracellular lipidic layer that covers the anther surface and protects anthers from external abiotic stresses, water loss from the inner tissues, and attack by pathogens [37,38]. It is composed of a cutin polymer matrix and waxes [37]. In plants, cuticular wax, cutin, and suberin are commonly defined as surface-covering lipids [37]. Cutin is a crosslinked, amorphous, and viscoelastic polymer formed almost exclusively by the interesterification of C16- and C18-polyhydroxy fatty acids [39]. Although the roles of AtLACS1 and AtLACS2 in anther and pollen development have not been reported, we may speculate the role of wheat orthologs of both genes in anther and pollen development. In this study, the expression of TaLACS1, TaLACS2, and TaLACS3 was detected at the three early stages. However, the expression of TaLACS1 was significantly higher at the two later stages. TaLACS2 and TaLACS3 were repressed at the vacuolated stage and induced at the binucleate and trinucleate stages under the cold condition. These results suggested that TaLACS2 and TaLACS3 may participate in wheat anther development. The expression of TaLACS17 was significantly higher than that of the cold-treated BS366 at the tetrad, binucleate, and trinucleate stages. TaLACS27 was significantly induced at the latter two stages, while TaLACS22 was only induced at the trinucleate stage in the cold-treated BS366 compared with the control-treated BS366. As the expression of the three LACS genes increased from the tetrad stage, we may conclude that the three genes may participate in late anther development and pollen exine formation. The differential expression of TaLACS17, TaLACS22, and TaLACS27 at the late stages suggest that male sterility in BS366 might have an effect on the fatty acid metabolism in the later anther development.

Three homoeologous genes, TaLACS16, TaLACS21, and TaLACS26 were clustered together with AtLACS8. TaLACS8, TaLACS9, TaLACS11, TaLACS13, TaLACS18, and TaLACS23 were clustered together with AtLACS9. AtLACS8 was reported to be functionally overlapped with AtLACS4 and AtLACS9 in the lipid trafficking between the ER and plastid for glycerolipid synthesis [1]. In this study, the expression of TaLACS11 and TaLACS16 was significantly higher at the tetrad stage in BS366 under the cold condition than that under the control condition. TaLACS8 were significantly induced at the uninucleate stage in the cold-treated BS366. Although AtLACS9 functions redundantly with either AtLACS1 or AtLACS4 in seed TAG biosynthesis, the differential expression of homologous wheat LACS in this study might suggest their roles in wheat male sterility [12,40]. Three LACS in Arabidopsis clustered together in the phylogenetic analysis, including AtLACS3, AtLACS4, and AtLACS5. The function of AtLACS5 is still unclear. It has been suggested that AtLACS5 is exclusively expressed in anthers [16]. GhACS1, an ortholog of the Arabidopsis AtLACS4 or AtLACS5, is predominantly accumulated in the anther and plays an essential role in microsporogenesis in the anther development of cotton [19]. TaLACS5 and TaLACS25, two orthologs of the AtLACS4 or AtLACS5, were differentially expressed in the BS366 anther under the cold and control conditions. The expression of TaLACS5 was repressed at the tetrad and vacuolated stages but induced at two latter stages in BS366 under cold conditions. TaLACS25 were induced at the binucleate stage in the cold-treated BS366. In addition to the plant cuticle, very-long-chain lipids were also detected in the extracellular pollen coat (trypine), where they play crucial roles.
in pollen-stigma communication [9]. Tryphine is mainly composed of complex lipids, wax esters, flavonoids, proteins, pigments, aromatic substances, and other unknown compounds [38, 41]. In the mature pollen grain, the tryphine fills the cavities of the pollen exine. Most lipids detected in the tryphine are derivatives of very-long-chain fatty acids (VLCFAs) [42]. It has been reported that LACS1 and LACS4 are required for proper pollen coat formation in Arabidopsis [9]. Thus, the differential expression of TaLACS5 and TaLACS25 may be correlated with pollen coat formation.

Male reproductive development in higher plants can be regulated by Gibberellins (GA). The transcriptional factor GAMYB is a crucial component of GA signaling in anther development. Transgenic barley over-expressing the HrGAMYB gene failed to dehisce and were male-sterile compared with non-transgenic controls [28]. It has been reported recently that the silencing of TaGAMYB in wheat displayed fertility decline and defects in tapetum, pollen and exine formation. In addition, either the hot temperature or GA3 treatment in YanZhan 4110S caused the down-regulation of TaGAMYB at the binucleate stage and trinucleate stage, as well as fertility decrease [43]. In this study, three orthologs of HrGAMYB were identified. The expression level of TaGAMYB-A was highest at the uninucleate and binucleate stages. The expression of TaGAMYB-B and TaGAMYB-D started to increase from the uninucleate stage and continued until the binucleate stage (Figure 6). All these results indicated that TaGAMYB-B and TaGAMYB-D might play critical roles in anther development from the uninucleate stage. All three TaGAMYB transcription factors were significantly repressed at the binucleate stage, which suggested their potential roles in the male sterility of BS366. The expression of TaGAMYB-B was negatively correlated with the expression of TaLACS4 and TaLACS5. The expression of TaLACS5 decreased from the binucleate stage. Differential expression analysis of the control- and cold-treated BS366 revealed that the expression of TaLACS5 was repressed under the cold condition at the tetrad and vacuolated stages. Thus, we may conclude that TaLACS5 might mainly function in anther development until the vacuolated stage. The repression of TaLACS5 at the vacuolated stage suggested the defect of BS366 under the cold condition impaired the lipid metabolism which involved the TaLACS5. In the cis-activating element analysis, MYB and MYC elements were found in all the promoter sequences of TaLACSs. Thus, we may conclude that wheat LACS genes including TaLACS5 may be regulated by the GAMYB transcription factors to participate in anther development.

4. Materials and Methods

4.1. Genome-Wide Identification of LACS Family Genes in Wheat

Nine LACS proteins in Arabidopsis were used in this study. Sequences of nine proteins were downloaded from the Arabidopsis genome database (https://www.Arabidopsis.org/) (accessed on 1 April 2022) [6]. The BLASTP method was used to identify the candidate LACS proteins in wheat with a cutoff e-value of $1 \times 10^{-20}$ and a score higher than 100 [44]. The HMM profile of the AMP-binding proteins (PF00501) was downloaded from the Protein family database (Pfam, https://pfam.xfam.org/) (accessed on 7 April 2022). The software HMMER was used to search for the AMP-binding proteins encoding genes against the genome of wheat using the HMM file of AMP-binding with a cutoff of $E < 1 \times 10^{-20}$. The conserved domain of the generated AMP-binding proteins in wheat were filtered using the Pfam, SMART (http://smart.embl.de/) (accessed on 8 April 2022), and CDD (https://www.ncbi.nlm.nih.gov/cdd/) (accessed on 10 April 2022) in NCBI. All retained proteins were blasted against the Arabidopsis genome to eliminate proteins with other functions. Finally, 30 genes were common to both methods. After excluding splice variants, the longest transcripts of 30 TaLACSs were retrieved from wheat. All identified wheat genes were renamed according to their genomic locations. Finally, the acquired sequences were submitted to ExPASy (https://web.expasy.org/protparam) (accessed on 11
April 2022)) to calculate the physicochemical parameters such as molecular weight (MW) and theoretical isoelectric point (pI).

4.2. Multiple Alignment and Phylogenetic Analysis of The Wheat LACS Family Genes

To determine the evolutionary relationships of the wheat LACS, the amino acid sequences of nine Arabidopsis LACS and 30 wheat LACS were subjected to sequence alignment and phylogenetic tree construction using MEGA7 software. The ClustalW function in MEGA7 was used for sequence alignment. An un-rooted neighbor joining (NJ) tree for LACS proteins in two species was constructed using MEGA7 [45]. An un-rooted neighbor joining (NJ) tree for LACS proteins only in wheat was also constructed in this research.

4.3. The Gene Structure and Conserved Domains in Wheat LACS

Fifteen conserved motifs with lengths of 6–50 amino acids were obtained for wheat LACS using meme-5.1.0 (https://meme-suite.org/meme/tools/meme (accessed on 7 July 2022)) [46]. Combined with the wheat genome annotation information, the conserved motifs and intron/exon pattern of the gene structure was determined for wheat LACS. The conserved domain and gene structure was analyzed and visualized with TBtools v1.098767 (https://github.com/CJ-Chen/TBtools/releases (accessed on 22 Aug 2022)) [47].

4.4. Chromosomal Location of TaLACS and Gene Duplication Analysis

To map the putative LACS genes onto the wheat chromosomes, the initial chromosomal position of TaLACSs and length of the chromosome were identified from the wheat genome (http://ftp.ensemblgenomes.org/pub/plants/release-54/gff3/triticum_aestivum/ (accessed on 7 July 2022)). Full amino acid sequences of TaLACSs were subjected to a multi-sequence blast, and the top four targets with an E-value lower than $1 \times 10^{-10}$ were selected for this study. The chromosomal distribution and collinearity between TaLACSs genes were visualized by TBtools v1.098767 (https://github.com/CJ-Chen/TBtools/releases (accessed on 22 Aug 2022)) [47]. Tandem duplication of events was defined as two or more adjacent homologous genes located on one chromosome without any intervening gene [22].

4.5. GO Annotation and Cis-Acting Elements Analysis of TaLACSs

The Gene Ontology (GO) annotations for wheat LACS were carried out using the TBtools [47]. The molecular functions, biological processes, and cellular components were annotated for the wheat LACS. Sequences of 2000 bp upstream of TaLACSs promoter were obtained [47]. The cis-acting elements in these regions were predicted using plantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 1 May 2022)) [48].

4.6. Expression Analysis of TaLACSs in Wheat Tissues

To study the expression patterns of all the TaLACSs, expression data from five wheat tissues (root, stem, leaf, spike, and grain) in Chinese Spring were obtained from WheatOmics 1.0 (http://202.194.139.32/ (accessed on 5 May 2022)). To further study the potential roles of TaLACSs involved in anther and pollen development, the transcriptome sequencing data of anthers at early uninucleate, vacuolated, binucleate, and mature pollen stages were used to examine the expression of all the putative TaLACSs in this study.

4.7. Phenotypic Analysis of BS366

The wheat temperature-sensitive genic male-sterile (TGMS) line Beijing Sterility 366 BS366, maintained at the Institute of Hybrid Wheat, Beijing Academy of Agriculture and Forestry Sciences, was used in this study. BS366 is normal at 20 °C (control) but produces sterile pollen at 12 °C (cold) with 12 h of daylight. Spikelets of BS366 from the uninucleate to trinucleate stages under the cold and control conditions were sampled and fixed in FAA
solution (formaldehyde: glacial acetic acid: 50% ethanol = 5: 5: 9). The pollens were dyed with improved carbol fuchsin solution, as previously reported [21]. Photographs of the microspores and pollen were obtained using an Olympus BX-53 microscope (Tokyo, Japan). For the anther phenotype analysis, the preparation of the transverse sections of anthers was carried out as reported [21]. The anther morphology was analyzed with a scanning electron microscope (HITACHI SU8100).

4.8. Plant Materials, Total RNA Isolation, and qRT-PCR Analysis

The anther transcriptome data of a normal inbred line CP1860 maintained at the Institute of Hybrid Wheat, Beijing Academy of Agriculture and Forestry Sciences was used in this study. Anthers of the wheat inbred line CP1860 were sampled at the early uninucleate, vacuolated, binucleate, and trinucleate stages, with three biological replicates. All samples were frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA). The concentration and quality was determined with a Nanodrop spectrophotometer and 1% agarose gel electrophoresis. All samples were sequenced using the MGISEQ-T7 platform. Raw reads were filtered to obtain high-quality reads, by removing low-quality reads containing more than 30% bases with Q < 20. After trimming low-quality bases (Q < 20) from the 5’ and 3’ ends of the remaining reads, the resulting high-quality clean reads in each sample were mapped onto the wheat reference genome using HISAT 2.2.1 release 7 (https://daehwankim-lab.github.io/htsat2/ (accessed on 7 Aug 2020) [49]. Only reads that could be mapped onto only one location in the reference genome (unique hits) were kept for further analysis. Fragments per kilobase of exon model per million reads (FPKM) was used to estimate the transcript expression levels in all samples.

For real-time qRT-PCR, cDNA was synthesized according to the manufacturer’s instructions (PrimeScript™ RT reagent Kit with gDNA Eraser, Takara Bio Inc., Shiga, Japan). The expression of TaLACSs was quantified with a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green II (Takara Bio Inc., Shiga, Japan). The expression levels of mRNAs in samples were normalized using the endogenous wheat actin gene with primer sequences 5′-TACTCCCTCAACAACCG-3′ and 5′-AGAACCTCCACTGAGAACAA-3′. The relative expression levels were calculated using the 2−ΔΔCt method. Primer sequences were designed using Primer3 input version 4.0 (http://primer3.ut.ee/ (accessed on 20 May 2022)). Primers for expression validation are listed in Supplementary Table S4.

5. Conclusions

In conclusion, a total of 30 LACS family genes were identified in the wheat genome. The chromosomal location, phylogenetic classification, gene structure, gene duplication, functional domains, and conserved motifs were investigated. The expression assay of all the candidate TaLACSs in five wheat tissues and anthers at different developmental stages were analyzed. The correlation of TaLACSs between TFs function in anther development was calculated. The expression of several TaLACSs were quantified in the cold- or control-treated BS366, a TGMS line. We may conclude that wheat orthologs of AtLACS2, AtLACS3, and AtLACS5 might participate in wheat anther development. Results presented here will be of great value to further characterize the biological roles of LACS in male fertility.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231911942/s1.

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