INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–0192/2020/24–4–863–870
DOI: 10.17957/IJAB/15.1510
http://www.fspublishers.org

Full Length Article

Genome-Wide Analysis of Long Chain Acyl-CoA Synthetase (*LACS*) Genes in Sunflower (*Helianthus annuus)* Suggests their Role in Drought Stress

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Received 05 February 2020; Accepted 13 May 2020; Published 16 August 2020

Abstract

Epicuticular wax acts as first line of defense to protect the areal parts of land plants from biotic and abiotic stresses. Genes belonging to long-chain acyl-CoA synthetase *(LCAS*) family are known to be involved in cuticular wax biosynthesis. However, very little is known about how *LACS* genes function during drought stress. As the sunflower genome has been recently sequenced, hence previously no genome-wide analysis about the cuticular wax biosynthesis genes has been conducted in this crop. We identified the *LACS* genes in sunflower by using different bioinformatics tools and characterized their relative expression under drought stress. Phylogenetic analysis divided thirty-five *Arabidopsis*, sunflower and maize *LACS* genes in seven subgroups. Our results of qRT-PCR analysis indicated that expression of *LACS* genes was upregulated under drought stress as compared to controls. So, it can be concluded that *LACS* genes play a role in adaptation to limited water conditions and can be exploited to imrove drought toleance. This research will lay a foundation for future studies about *LACS* gene family in sunflower.

Keywords: Cuticular wax; *LACS*; Drought stress; Wax biosynthesis; Plant lipids

Introduction

Sunflower (*Helianthus annuus*) belongs to compositae family and is native to North America (Schilling and Heiser 1981; Blackman *et al*. 2011). This crop includes diploid, tetraploid and hexaploid species having basic set of chromosomes 17 (Rieseberg and Seiler 1990). It is an important oil seed crop and complementary source of protein for human being, dairy and livestock animals. Sunflower is also used for ornamental purpose and is a source of chemical feed stock. Sunflower genome was sequenced in 2017 and reportedly has estimated genome size of 36 gigabases (Badouin *et al.* 2017).

Cuticular waxes are mixture of long chain fatty acids and their derivatives (Shaheenuzzamn *et al*. 2019; Alfarhan *et al*. 2020). Basic components of plant cuticular waxes are aldehydes, alkanes, fatty acids, ketones, acetones, wax esters, terpenoids and sterols (Shaheenuzzamn *et al*. 2019). Biochemical mechanism of wax elongation is fully characterized, however very less information is available about the proteins involved in wax biosynthesis process after 26 carbons (Pascal *et al*. 2013). Cuticular wax seals the areal parts of land plants to prevent them from non-stomatal water loss (Ahmad *et al.* 2015; Alfarhan *et al*. 2020). Leaf glaucousness is a trait referred to as plants adaptation to drought (Islam *et al.* 2009). Reduction in wax quantity exhibits high transpiration rate where as high water loss has been observed in low waxy leaves (Alfarhan *et al*. 2020). Cuticular waxes resist plants against insects, pathogens and bacteria (Zeisler-Diehl *et al.* 2018), protect plants from ultraviolet radiations (Laila *et al.* 2017; Alfarhan *et al.* 2020), decrease water deposition on plant surface, reduce the retention of dust, air pollutants and pollens (Wang *et al*. 2019). Wax biosynthesis process starts with elongation of 16:0 Acyl-CoA to very long chain fatty acid which is reduced to primary alcohol and formation of alkyl ester (Lai *et al*. 2007).

Long-chain acyl-CoA synthetase (*LACS*) has a critical role in biosynthesis of all fatty acid derived molecules particularly in cuticular wax and cutin biosynthesis pathways (Lü *et al*. 2009). *LACS* esterifies free fatty acids to acyl-CoAs, a key activation step that is necessary for the utilization of fatty acids by most lipid metabolic enzymes (Lü *et al*. 2009; Pulsifer *et al.* 2012). Sometime LACS enzyme expresses dual role in activation of long chain fatty acids for synthesis of cellular lipids and their

To cite this paper: Ahmad HM, Mahmood-ur-Rahman, F Azeem, T Shaheen, MA Irshad (2020). Genome-wide analysis of long chain acyl Co-A synthetase (*LACS*) genes in sunflower (*Helianthus annuus)* suggests their role in drought stress. *Intl J Agric Biol* 24:863–870

degradation *via* beta-oxidation (Jenks *et al*. 1995). Biosynthesis mechanism of cuticular waxes begins with the synthesis of C16 and C18 long chain fatty acids (LCFA) in plastids (Ahmad *et al.* 2015; Shaheenuzzamn *et al*. 2019). These LCFAs are then transported to cytoplasm where coenzyme (CoA) activate them by long chain acyl-CoA synthetases (Schnurr *et al*. 2004; Samuels *et al.* 2008).

Cuticular wax is a lipid-based barrier to seal the areal surface of land plants and play protective role (Alfarhan *et al*. 2020). *LACS1* gene functions as very long chain acyle-CoA synthetase during wax metabolism (Lü *et al*. 2009). Similarly, *LACS2* gene have overlapping function with *LACS1* in cutin and wax synthesis (Pulsifer *et al*. 2012). In *A. thaliana*, *LACS1*, *LACS2* and *LACS3* are expected to be cuticle biosynthesis genes (Pulsifer *et al*. 2012). They are also expressed for intracellular trafficking and transmembrane transport (Pulsifer *et al*. 2012). *LACS6* gene acts in both the wax and cutin biosynthesis pathways preferentially uses palmitoleate, palmitate, linoleate and eicosenoate (Lü *et al.* 2009) and seems to have a specific activity against very long-chain fatty acid (VLCFA) class with acids longer than 24 carbons (Lü *et al.* 2009). *LACS6* also show redundant function when it expressed with *LACS7* during seed development process (Shockey *et al.* 2002).

The aim of present study was genome-wide analysis of *LACS* gene family in sunflower. Further genomic comparison was performed between *Arabidopsis* and sunflower to find the functional similarities in them by using different bioinformatics tools. To explore the role of *LACS* family for wax biosynthesis genes in sunflower, we subjected the sunflower genotypes to drought stress and their expression profile was studied.

Materials and Methods

Retrieval of protein sequences

Protein sequences of *A. thaliana LACS* genes were retrieved from "The *Arabidopsis* Information Resource" (TAIR) (https://www.arabidopsis.org/). As, no *LACS* gene has been characterized in *H. annuus*, hence pblast program at NCBI (https://www.ncbi.nlm.nih.gov/) was used to obtain the similar sequences in this crop. These sequences were further verified at Plant Genome and System Biology (PGSB) databases (https://pgsb.helmholtz-muenchen.de/plant/plantsdb.jsp) and Phytozome v. 11.0 (https://phytozome.jgi.doe.gov/pz/portal.html) database.

Physio-chemical properties of LACS proteins and subcellular locations

Different physio chemical properties such as exon numbers, amino acid length, molecular weight, isoelectric point of LACS proteins in *A. thaliana* and *H. annuus* were computed by using online web tool "protpram" on Expasy (https://web.expasy.org/cgi-bin/protparam/protparam) according to Gasteiger *et al*. (2005). Sub cellular location of *LACS* genes in *Arabidopsis* and sunflower was determined according to Chou and Shen (2010) using server (Plant-mPLoc https://www.csbio.sjtu.edu.cn/bioinf/plant/).

Sequence alignment and construction of phylogenetic tree

LACS protein sequences of *A. thaliana*, *H. annuus* and *Z. mays* were aligned by online tool ClustalX (Sun *et al*. 2015). Aligned sequences were used for construction of phylogenetic tree according to neighbor joining method (Saitou and Nei 1987) by using MEGA 5.2 program (Tamura *et al*. 2011) at 1000 boost strap value.

Conserved motifs, gene structure analysis and chromosomal mapping

To further study the structure of LACS proteins, conserved motif analysis of LACS proteins was carried out by using MEME SUIT 4.9.1 tool (https://meme.nbcr.net/meme/cgi-bin/meme.cgi) with their default parameters. Intron and exon organization in both plant species was discovered by using Gene Structure Display Server (GSDS) 2.0 (https://gsds.cbi.pku.edu.cn/). Chromosomal mapping of *A. thaliana* genes were performed by using chromosome map tool at The *Arabidopsis* Information Resource (TAIR) (https://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp) whereas in *H. annuus* exact location of *LCAS* genes were mapped by using excel sheet.

Prediction of evolutionary history and protein-protein interaction

To predict the evolutionary history of these genes, protein sequences of *A. thaliana* and *H. annuus* were submitted to online synteny tool Circoletto (tools.bat.infspire.org/circoletto). The predicted protein–protein interaction (PPI) map of LACS proteins was generated from the STRING database (https://string-db.org/cgi/my.pl?sessionId=1Ye2FGXxwMVL) (Szklarczyk *et al*. 2011).

Plants material and drought treatment

To analyze the expression pattern of *HanLACS1* and *HanLACS3* genes in sunflower under drought conditions, four sunflower genotypes Hysun-33, FH-331, FH-629 and FH-630 obtained from Oilseed Research Institute, Ayyub Agriculture Research Institute, Faisalabad, were cultivated in pots in growth chambers, containing red sandy soil and manure (2:1) with a program set to 25/22°C (day/night), 16-h photoperiod, and relative humidity of 75%. At the age of 30 days' plants were subjected to drought stress by withholding water for ten days. Samples from both treated and non-treated plants were collected for three biological

replicates and were frozen immediately in liquid nitrogen at -80°C until further analysis.

RNA isolation and RT-qPCR analysis

Total RNA from frozen samples was isolated by using TriZol reagents according to the manufacturer's instructions. RNA concentration was measured with nanodrop, ND-1000 (Nano Drop Technologies, Inc.), spectrophotometer using the nucleic acid program. Primers were designed from a list of genes belonging to *LACS* gene family involved in epicuticular wax biosynthesis based on previous studies by using online tool primer3 (https://frodo.wi.mit.edu/). Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used for first strand cDNA synthesis. Gene expression levels were studied by quantitative real time PCR using SYBER Green qPCR Master Mix (ThermoFisher Scientific, U.S.A.) in CFX96 Real-Time PCR System (BIO-RAD, U.S.A.). The variations in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ analysis method. The quantification was carried out by the *Actin* gene as a reference gene. The specific primers for *LACS* genes used in qPCR could be seen in Table 1.

Results

Identification of *LACS* genes, physio-chemical properties and subcellular locations

Previously *LACS* family has not been characterized in sunflower due to un-availability of genome sequence. Information about the physiochemical characteristics of *A. thaliana* are shown in Table 2. Chromosomal number indicated that *LACS* genes were present on all the five *Arabidopsis* chromosomes and exon number varied from 12 to 23. It was observed that length of genomic DNA was ranged from 3480 bp (LACS8-2) to 5609 bp (LACS6-1). The number of amino acids ranged from 522 (LACS2-2) to 720 (LACS8-1, LACS8-2). Predicted molecular weight varied from 78342.6 to 57634.5. The theoretical isoelectric point was ranging from 5.63 to 8.01 indicating that some proteins are basics, and some were acidic in nature. Subcellular location showed that out of sixteen proteins six were present in endoplasmic reticulum, six in chloroplast, one each in plasma membrane, nucleus, golgi apparatus and mitochondria.

In *H. annuus*, exon numbers were counted from 5 (LACS3-1) to 23 (LACS6-1). Amino acid length of LACS proteins in this crop species varied from 95 (LOC110868952) to 720 (HannXRQ_Chr04g0126391). Genomic length was in range of 1117bp to 18099 bp (Table 3). Molecular weight of proteins was diversified from 10985.88 kDa to 78573.77kDa. Isoelectric point of sunflower LACS proteins (pI) was in between from 5.37 to 8.49. subcellular location indicated that seven proteins were present in chloroplast, five in plasma membrane, four in golgi apparatus, and two in nucleus and one in peroxisomes.

Phylogenetic relationship of LACS proteins in *A. thaliana* and *H. annuus*

To study the phylogenetic relationship of LACS proteins in *A. thaliana, H. annuus and Z. mays* a phylogenetic tree was constructed by multiple sequence alignment of these proteins (Fig. 1). Phylogenetic tree divided LACS proteins in seven subgroups. These clusters contained 13, 8, 12, 8, 7, 4 and 5 members respectively. First cluster was the largest than others with thirteen members and sixth was the smallest. It was observed that first five clads contained protein members from all three species suggesting that these proteins are evolutionary conserved. Our results are in line with (Azeem *et al*. 2018; Waqas *et al*. 2019) who reported similar results in chickpea. 6th clad possessed only *Arabidopsis* proteins and 7th clad was belonging to *H. annuus* proteins only which mean that these proteins may be evolutionary diverse from each other.

Gene structure analysis of *LACS* genes in *A. thaliana* and *H. annuus*

To get the information regarding intron and exon organization, their number and length in LACS gene families of *A. thaliana* and *H. annuus*, genomic DNA and coding sequences were analysed by using GSDS 2.0 server. The results revealed that intron-exon structures are conserved within groups of *LACS* genes (Fig. 2). Maximum number of exons was present in *HaLACS6-1* which was 23. According to intron-exon length *HaLACS6-3* was the smallest gene which contained only three exons. Further it was observed that some genes clustered together having similar numbers and length of CDSs, even they showed variation in length of introns and untranslated regions (UTRs). Two genes, *HaLACS8-2* and *HaLACS9-2* were closely related to each other as they fall in same cluster and have similar nature of intron-exon organization throughout the genome. Similarly, *AtLACS8-1*, *AtLACS7-1* and *AtLACS6-1* have homology among them and fall in same group. Similarity in intron/exon organization within a subgroup of phylogenetic tree has been reported by (Bari *et al*. 2018; Waqas *et al*. 2019).

Conserved motif analysis for LACS proteins in *A. thaliana* and *H. annuus*

The results of conserved motifs were presented in Fig. 3. Motif analysis showed ten different conserved motifs in *Arabidopsis* and sunflower in LACS proteins. Maximum 11 motifs were recorded on *Arabidopsis* protein LACS 6-1 while minimum 1 motif was noticed on sunflower protein LACS6-2. It was also noted that pattern of conserved motifs was almost same with in a clad of phylogenetic tree. As previously no conserved motif analysis was available in sunflower hence, we were unable to compare our results.

Table 1: List of primers used for qRT-PCR

S. No.	Primer type and name	Sequence (5´-3´)
1.	Forward primer for LACS1	ACTGCTTGGGACATTTTCAG
	Reverse primer for LACS1	TCCATTGCTATGATCCACTG
2.	Forward primer for LACS3	TCAGTTCCAGAGATGGGTTA
	Reverse primer for LACS3	AGATGTTCTTCTTACGGTCG
3.	Forward primer for Actin	TCATGAAGATCCTGACGGAG
	Reverse primer for Actin	AACAGCTCCTCTTGGCTTAG

Table 2: Different Physio-chemical characteristics of *LACS* genes and their homologues showing variability in *A. thaliana*

S. No	Gene symbol	Gene ID	Locus tag	Ch. No	Exon	a. a	G.L bp	Protein M.W kDa	PI	Sub. Cel. Location
1	LACS1-1	819337	AT2G47240.1	2	19	660	5411	74597.4	6.3	E. R
2	LACS1-2	819337	AT2G47240.2	2	19	660	5088	74597.4	6.3	E.R
3	LACS1-3	819337	AT2G47240.3	2	19	660	4248	74597.4	6.3	E.R
4	LACS1-4	819337	AT2G47240.4	2	19	601	3726	68143.1	6.4	E.R
5	LACS2-1	841367	AT1G49430.1	1	19	665	4964	74388.5	6.02	Ch. p
6	LACS2-2	841367	AT1G49430.2	1	19	522	3666	57634.5	6.07	Ch. P
7	LACS3-1	842748	AT1G64400.1	1	17	665	4266	74750.6	7.71	E.R
8	LACS3-2	842748	AT1G64400.2	1	17	663	4724	74112.8	8.01	E.R
9	LASC4-1	828484	AT4G23850.1	4	19	666	4968	74507.2	5.63	P. Mem
10	LASC5-1	826704	AT4G11030.1	4	19	666	4357	74063.7	6.99	Ch. P
11	LACS6-1	819767	AT3G05970.1	3	23	701	5609	76602.7	8.01	Ch. P
12	LACS7-1	832820	AT5G27600.1	5	23	700	4533	77352.6	6.56	Nuc.
13	LACS8-1	814974	AT2G04350.1	2	12	720	3675	78342.6	7.7	Mito. C
14	LACS8-2	814974	AT2G04350.2	2	12	720	3480	78342.6	7.7	Gol. A
15	LACS9-1	844094	AT1G77590.1	1	12	691	4236	76175.1	6.97	Ch. p
16	LACS9-2	844094	AT1G77590.2	1	12	545	4109	59721.5	7.66	Ch. p

Where E.R= Endoplasmic reticulum, Ch.P= Chloroplast, P.Mem= Plasma membrane, Nuc= Nucleus, Mito.C= Mitochondria and Gol.A= Golgi apparatus

Table 3: Different Physio-chemical characteristics of *LACS* genes and their homologues showing variability in *H. annuus*

S. No	Gene symbol	Gene ID	Locus tag	Ch. No	Exon	a.a	G.L bp	Protein M.W	PI	Sub Cel. location
1	LACS1-1	110910053	HannXRQ_Chr15g0465101	15	20	661	5263	74762	7.2	Ch.P
2	LACS1-2	110936374	HannXRQ_Chr04g0106251	4	19	659	7782	74673	5.8	Ch.P
3	LACS2-1	110886089	HannXRQ_Chr10g0307531	10	19	659	9759	73483	5.7	Ch.P
4	LACS3-1	110901830	HannXRQ_Chr13g0415221	13	5	265	1648	29292	5.9	Ch.P
5	LASC4-1	110930246	HannXRQ_Chr03g0083551	3	18	661	8502	73504	6.1	P.Mem
6	LACS4-2	110867977	HannXRQ_Chr07g0190911	7	19	659	6911	73500	6.5	P.Mem
7	LACS4-3	110899500	HannXRQ_Chr13g0415231	13	19	660	6911	73797	8.5	P.Mem
8	LACS4-4	110899498	HannXRQ_Chr13g0415151	13	19	660	6823	73818	7	P.Mem
9	LACS4-5	110931053	HannXRQ_Chr03g0093411	3	19	664	4920	73987	6.7	P.Mem
10	LASC6-1	110885243	HannXRQ_Chr10g0296521	10	23	697	7263	76349	7.5	Ch.P
11	LACS6-2	110872567	HannXRQ_Chr08g0220471	8	9	175	2630	19693	5.7	Ch.P
12	LACS6-3	110868952	LOC110868952	7	3	95	1117	10986	8.5	Ch.P
13	LACS7-1	110929342	HannXRQ_Chr03g0071661	3	22	698	9942	76955	6.6	Nuc.
14	LACS7-2	110867086	LOC110867086	7	11	282	3739	31359	5.4	Per.Oxi
15	LACS7-3	110895747	HannXRQ_Chr01g0009391	1	17	315	7029	35712	5.8	Nuc.
16	LACS8-1	110938092	HannXRQ_Chr04g0126391	4	12	720	7440	78574	7.9	Gol.A
17	LACS8-2	110889499	HannXRQ_Chr11g0326022	11	12	697	18099	76302	7.1	Gol.A
18	LACS9-1	110878808	HannXRQ_Chr09g0260621	9	12	696	5345	76226	6.1	Gol.A
19	LACS9-2	110889498	HannXRQ_Chr11g0326021	11	12	697	18099	76302	7.1	Gol.A

Where, Ch.P= Chloroplast, P.Mem= Plasma membrane, Nuc= Nucleus, Per.Oxi= Peroxisome and Gol.A= Golgi apparatus

Chromosomal mapping of *LACS* genes in *A. thaliana* and *H. annuus*

The results of Fig. 4 (a) indicated that in *Arabidopsis*, *LACS* genes were present on all the five chromosomes. Maximum three genes were present on different locations at 1st chromosome. In sunflower genome *LACS* genes were unevenly distributed on seventeen chromosomes. It was noted that maximum four genes were located on 7th chromosome however no *LACS* gene was present on chromosome 2, 5, 12, 14, 16 and 17. Distribution of *LACS* genes in both plant species is showen in Fig. 4 (a, b).

Evolutionary relationship of *Arabidopsis* and Sunflower *LACS* genes

Evolutionary relationship among different species showed that weather these species were from same origin or not. It also enables us to know about their origin and ecosystem. High similarity indicates that these species were from same origin and similar environmental conditions whereas low similarity represents the contrasting environmental conditions. A comparative synteny analysis was performed to get the idea about the evolutionary relationship and `origin of *LACS* gene family in *Arabidopsis* and Sunflower.

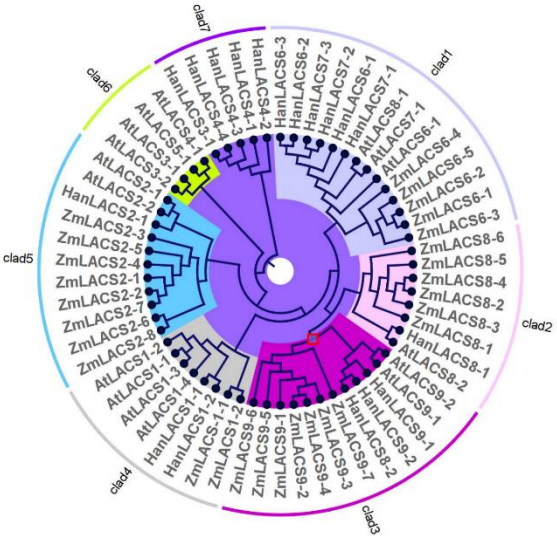

Fig. 1: Phylogenetic tree of LACS proteins. The tree was constructed with amino acid sequences of *A. thaliana, H. annuus and Z. mays* using neighbor joining method at boost strap value of 1000 replicates. Sequences were aligned with ClustalX and tree was constructed using MEGA 5.2 program

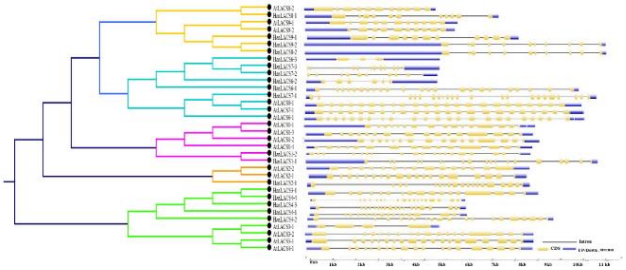

Fig. 2: Phylogenetic relationship and intron-exon organization of *A. thaliana* and *H. annuus LACS* genes. Yellow and blue boxes representing CDS and UTRs respectively and introns are represented by black lines. The analysis was performed by GSDS 2.0

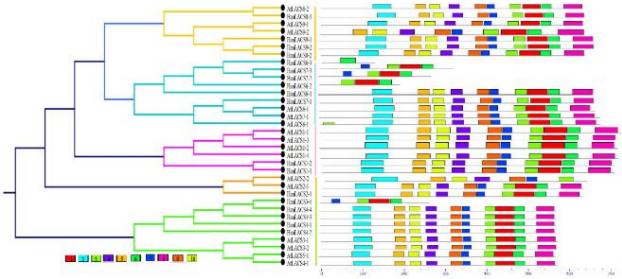

Fig. 3: Motif analysis of *A. thaliana* and *H. annuus LACS* genes. The colored box in each line represent motif. The non-conserved motifs are represented by blank lines

Synteny analysis was performed by using 16 *Arabidopsis* and 19 sunflower LACS proteins. The results showed that both these species were closely related to each other in their origin as per Fig. 5. *Arabidopsis LACS* gene *AtLACS9-1*

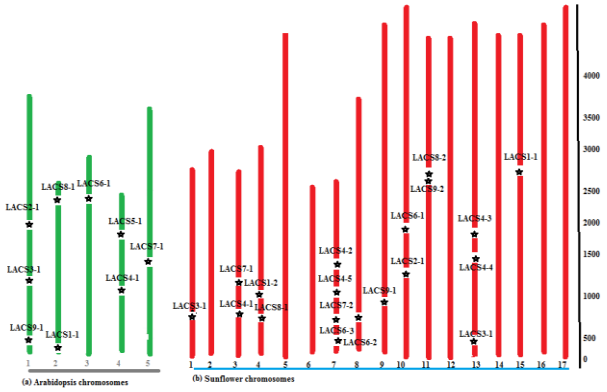

Fig. 4: Chromosomal mapping of *LACS* gene family in *A. thaliana* and *H. annuus.* Asterisks indicate the positions of genes on each chromosome

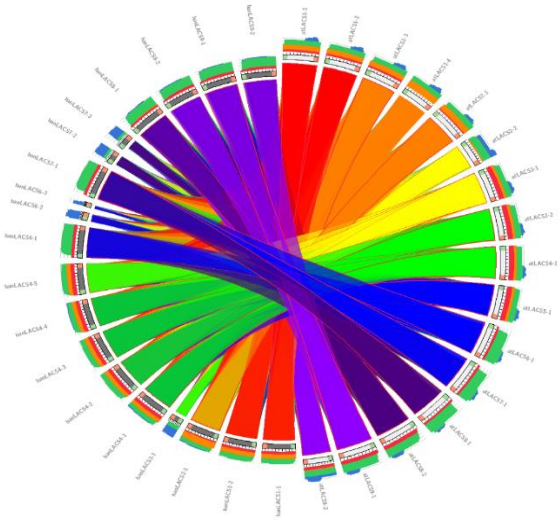

Fig. 5: Synteny analysis of *LACS* genes between *Arabidopsis* and sunflower. Colored lines which connect two regions indicate syntenic regions between *Arabidopsis* and sunflower

syntenic to sunflower *HaLACS9-1*, *HaLACS9-2*, *HaLACS8-1* and Ha*LACS8-2*. Similarly, *Arabidopsis LACS1*, *LACS2* were syntenic to sunflower *LACS1* and *LACS2* respectively. As previously no study was available in this regard hence results remained un-compared.

Prediction of LACS protein–protein interaction network

Protein-protein interaction analysis was carried out to reveal the unforeseen and unique functional role of well characterized protein. This interaction also explored that at which conditions these proteins interact and what are the functional implications of these interactions. So, to clarify the interaction among LACS1, LACS2, LACS3, LACS4, LACS5, LACS6, LACS7, LACS8 and LACS9 proteins in *Arabidopsis*, protein–protein interaction network was predicted using STRING online tool. The results (Fig. 6)

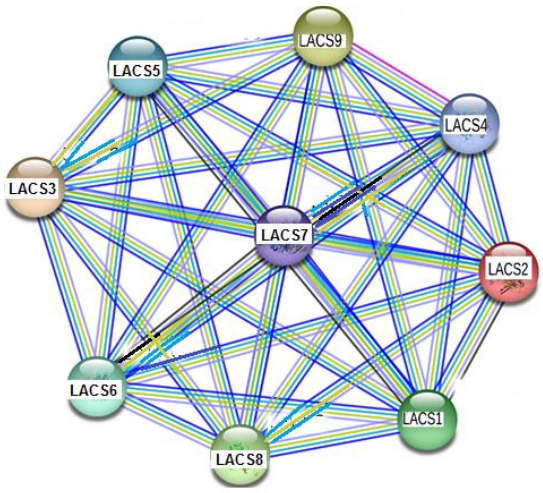

Fig. 6: Protein-protein interaction analysis of LACS proteins. The analysis was carried out using online server STRING

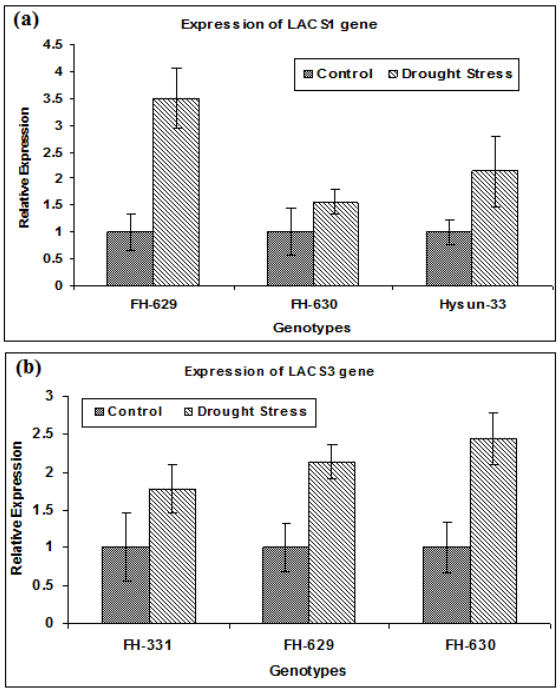

Fig. 7: Effects of drought stress on the expression of *LACS1* and *LACS3* in sunflower. The expression levels were examined by qRT-PCR. The results are means of three biological replications. Untreated plants were used as control

showed nine number of nodes, 36 number of edges, average node degree was 8, average local clustering coefficient was one and PPI enrichment *P*-value:< 1.0e-16.

Expression analysis of *LACS1* and *LACS3* genes in sunflower

We performed the Quantitative RT-PCR analysis to detect the expression of *LACS* genes in four sunflower cultivars by subjecting these cultivars under drought stress. Then expression of these genes was compared with normally watered plants. Expression analysis of *LACS* genes indicated that these genes showed their expression in all the four cultivars. *LACS1* showed its expression in three (FH-629, FH-630 and Hysun-33) cultivars, whereas *LACS3* expressed in (FH-331- FH-629 and FH-630) as shown in Fig. 7. It was noticed that drought stress upregulated the expression of under study *LACS* genes as compared to control. Higher expression of wax biosynthesis genes under drought stress has been observed in *Arabidopsis* (Seo *et al.* 2011), rice (Zhou *et al.* 2013) and wheat (Bi *et al.* 2017; Zhao *et al*. 2018).

Discussion

Biotic and abiotic stresses badly effect the growth and development of crop plants causing huge losses to grain yield (Zhou *et al.* 2020). Cuticular waxes play important role to protect the plants from various biotic and abiotic stresses such as, drought, salinity, cold, ultraviolet radiation, insects, bacteria and pathogens (Ahmad *et al.* 2015; Shaheenuzzamn *et al*. 2019). Wax biosynthesis gene families *i.e*., *LACS*, *CER*, *KCS*, *KCR* and *FAR* play important role in biotic and abiotic stress tolerance (Ahmad *et al*. 2015; Shaheenuzzamn *et al*. 2019). The role of these gene families has been characterized in *Arabidopsis*, rice, wheat and maize (Schnurr *et al*. 2004; Zhu *et al*. 2014; Shaheenuzzamn *et al*. 2019). However, no study was regarding sunflower. So, we selected the long chain acyl-CoA synthetase (*LACS*) gene family to validate its expression in sunflower under drought conditions. LACS converts free fatty acids to acyl-CoA thioesters that play an important role in fatty acid metabolism (Shockey *et al*. 2002). The other major function of LACS enzyme is fatty acid transportation. LACS enzymes are also involved in various fatty acids derived metabolic pathways *i.e*., fatty acid β-oxidation, triacylglycerol, phospholipids and jasmonate biosynthesis (Shockey *et al*. 2002).

In present research, we provided the complete overview of *LACS* gene family in sunflower. We conducted in silico identifications and explored the potential role of *LACS* genes in sunflower through computational tools. Further we analyzed the phylogenetic relationship, subcellular location, gene structure, chromosomal location, conserved motifs, and protein-protein interactions, along with expression analysis of *LACS* gene family under drought conditions. In previous research nine genes of this family *AtLACS1* to *AtLACS9* have been reported and characterized in *Arabidopsis* (Shockey *et al*. 2002). By BLAST analysis of *A. thaliana LACS* genes, we identified 19 *LACS* genes along with their small variants in sunflower. Subcellular localization analysis revealed that these genes were present in various cell organelles *i.e*., endoplasmic reticulum, nucleus, chloroplast, peroxisomes and mitochondria (Table 3).

Presence of LACS proteins in various key organelles of plant cell is the indication of their active participation in cellular metabolism during a biotic stress conditions (Carther *et al*. 2019). Previously presence of *LACS* genes in various subcellular locations has been reported by (Browse and Somerville 1991; Shockey *et al*. 2002; Fulda *et al*. 2004). A phylogenetic analysis is helpful to understand the evolutionary pattern of many morphological and chemical traits (Soltis and Soltis 2000, 2003). Our phylogenetic analysis gave rise to seven distinct clads (Fig. 1). First cluster was the most complex one and it was observed that *HanLACSs* were similar to other plant genes in this cluster. It was noted that *Arabidopsis*, sunflower and maize *LACS6*, *LACS7* genes were falling in same clad and *LACS3, LACS4* and *LACS5* in same subgroup which supported the results of (Fulda *et al*. 2004), who reported similar results in *Arabidopsis*.

The exon-intron distribution can be considered as an imprint of evolution in a gene family, which can provide extra evidence to reveal the phylogenetic relationship of the gene family from different organisms (Yang *et al*. 2019). In this study *LACS* genes from *Arabidopsis* and sunflower showed 3–18 introns (Fig. 2), indicating high structural diversity regarding *LACS* genes in these plants. During the comparison intron/exon organization of *LACS* gene between *Arabidopsis* and sunflower plants, it was observed that genes falling in same cluster probably have same exon numbers. Similarity between genes of different species showed that they came from similar ancestor and these genes were strongly affected by repetitive DNA duplication phenomena during the evolution process (Carther *et al*. 2019; Lynch and Conery 2000). Chromosomal mapping indicate that these genes were located on chromosomes 1, 2, 3, 6, 7, 8, 9, 10, 12 and 14 (Fig. 4).

Among abiotic stresses, drought is a major limiting factor that effect the plant growth, development and ultimately reduction in plant production (Awan *et al*. 2015; Javed *et al*. 2016). Many studies have been conducted to improve the plants adoptability to drought stress viewing root architecture, leaf organization, drought tolerance and avoidance mechanisms, however no prior concentration has been given for the identification of cuticular wax biosynthesis genes and their role under limited water conditions. Expression profile of two *LACS* genes *LACS1* and *LACS3* was determined in four sunflower cultivars by using qRT-PCR and their transcript level was determined under drought and normal conditions. *LACS1* showed its expression in three cultivars *i.e*., FH-29, FH-30 and Hysun-33 and *LACS3* expressed in FH-331, FH-629 and FH-630. It was observed that expression level of these genes was higher in drought stressed plants as compared to controls which were upregulated under drought conditions and have role in drought tolerance.

Previously, it has been reported that *Arabidopsis* and yeast *LACS1* genes are involved in wax biosynthesis, metabolic pathway, fatty acid and glycerol-lipid metabolism (Shockey *et al*. 2002; Lü *et al*. 2009; Pulsifer *et al*. 2012). *LACS1* gene function as very long chain acyl-CoA synthetase during wax metabolism (Kunst and Samuels 2003). Similarly, *Arabidopsis LACS3* gene have overlapping function with *LACS1* during cutin and wax synthesis (Lü *et al*. 2009). Variation in *LACS* gene expression under drought conditions showed their metabolic activities in plant tissues. Similar type of trend for several metabolites was noted in tomato (Zgallai *et al*. 2005), maize (Guo *et al*. 2017) and soybean (Carther *et al*. 2019).

Conclusion

Comprehensive genomic analysis and expression profiling of *LACS* genes in *H. annuus* revealed the presence of 19 genes in this species. These genes were located in different chromosomes and were present in various subcellular locations. Phylogenetic and conserved motif analysis confirmed the evolutionary association among sunflower and *Arabidopsis LACS* genes. Results of qRT-PCR showed that drought stress upregulated the expression of under study *LACS* genes as compared to control. This information would be helpful for selection of wax and stress responsive candidate genes in sunflower for further studies. Moreover, this is among the pioneer investigations on comparative genomics of wax biosynthesis genes in sunflower that may lead the foundation for further research in this aspect.

Acknowledgements

The authors are thankful to Mr. Fida Hussain, Research Officer, Oilseed Research Institute, Ayyub Agriculture Research Institute, Faisalabad, Pakistan, for the provision of sunflower genotypes to conduct the research.

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