Article

Not peer-reviewed version

Expression of *FAD* and *SAD* Genes in Developing Seeds of Flax Varieties under Different Growth Conditions

Elena N. Pushkova , Liubov V. Povkhova , Ekaterina M. Dvorianinova , Roman O. Novakovskiy , Tatiana A. Rozhmina , Aleksey A. Gryzunov , Elizaveta A. Sigova , Daiana A. Zhernova , Elena V. Borkhert , Anastasia A. Turba , Arthur G. Yablokov , Nadezhda L. Bolsheva , Alexey A. Dmitriev * , Nataliya V. Melnikova *

Posted Date: 13 December 2023

doi: 10.20944/preprints202312.0947.v1

Keywords: Linum usitatissimum; flax; fatty acids; SAD; FAD; gene expression; transcriptome sequencing

Article

Expression of *FAD* and *SAD* Genes in Developing Seeds of Flax Varieties under Different Growth Conditions

Elena N. Pushkova [1,†], Liubov V. Povkhova [1,†], Ekaterina M. Dvorianinova [1,2], Roman O. Novakovskiy [1], Tatiana A. Rozhmina [1,3], Aleksey A. Gryzunov [4], Elizaveta A. Sigova [1,2], Daiana A. Zhernova [1,5], Elena V. Borkhert [1], Anastasia A. Turba [1], Arthur G. Yablokov [1], Nadezhda L. Bolsheva [1], Alexey A. Dmitriev [1,*] and Nataliya V. Melnikova [1,*]

[1] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia; pushkova18@gmail.com (E.N.P.); povhova.lv@phystech.edu (L.V.P.); dvorianinova.em@phystech.edu (E.M.D.); 0legovich46@mail.ru (R.O.N.); sigova.ea@phystech.edu (S.E.A.); zhernova.d@ya.ru (D.A.Z.); sashai@inbox.ru (E.V.B.); anastas.turba@gmail.com (A.A.T.); melarsoprol@mail.ru (A.G.Y.); nlbolsheva@mail.ru (N.L.B.); alex_245@mail.ru (A.A.D.); mnv-4529264@yandex.ru (N.V.M.);

[2] Moscow Institute of Physics and Technology, 141701 Moscow, Russia;

[3] Federal Research Center for Bast Fiber Crops, 172002 Torzhok, Russia; tatyana_rozhmina@mail.ru (T.A.R.);

[4] All-Russian Scientific Research Institute of Refrigeration Industry – branch of V.M. Gorbatov Federal Research Center for Food Systems of Russian Academy of Sciences, 127422 Moscow, Russia; grizunov@rambler.ru (A.A.G.);

[5] Faculty of Biology, Lomonosov Moscow State University, 119234 Moscow, Russia;

* Correspondence: alex_245@mail.ru (A.A.D.); mnv-4529264@yandex.ru (N.V.M.)

† These authors contributed equally to this work.

Abstract: Flax seed is one of the richest plant sources of linolenic acid (LIN) and also contains unsaturated linoleic acid (LIO) and oleic acid (OLE). Stearoyl-ACP desaturases (SAD) and fatty acid desaturases (FAD) play a key role in the synthesis of flax fatty acids (FA). We sequenced flax seed transcriptomes at the 3, 7, 14, 21, and 28 day after flowering (DAF) for ten flax varieties with different oil FA compositions grown under three temperature/watering conditions. Expression levels of 25 genes of *SAD*, *FAD2*, and *FAD3* families were evaluated. *FAD3b*, *FAD3a*, *FAD2b-2*, *SAD3-1*, *SAD2-1*, *SAD2-2*, *SAD3-2*, *FAD2a-1*, and *FAD2a-2* had the highest expression levels, which changed significantly during seed development. These genes probably play a key role in the FA synthesis in flax seeds. High temperature and insufficient watering shifted the maximum expression levels of the *FAD* and *SAD* genes to earlier development stages, while the opposite trend was observed for low temperature and excessive watering. Differences in *FAD* and *SAD* expression profiles under different growth conditions could contribute to the FA composition of linseed oil. Stop codons in the *FAD3a* gene, resulting in reduced LIN content, decreased the level of the *FAD3a* transcript. Some difference in reaching the maximum expression level during seed development was observed between 1) *SAD3-1*, *SAD3-2*, and *FAD2b-2* and 2) *SAD2-1*, *SAD2-2*, *FAD2a-1*, *FAD2a-2*, *FAD3a*, and *FAD3b.* These groups possibly contribute somewhat differently to the synthesis of FA at different development stages.

Keywords: *Linum usitatissimum*; flax; fatty acids; *SAD*; *FAD*; gene expression; transcriptome sequencing

1. Introduction

Flax (*Linum usitatissimum* L.) seed is one of the richest plant sources of omega-3 fatty acids, which are essential for humans and prevent the onset and progression of many diseases [1–4]. Flax varieties differ significantly in the fatty acid (FA) composition of the oil, especially in the content of linolenic acid (omega-3) [5–7]. Traditional flax varieties are high in linolenic acid (LIN, 50-60%), and are used

 doi:10.20944/preprints202312.0947.v1

in the pharmaceutical and paint industries [6,8]. Varieties with low (about 5%) and medium (30-40%) LIN content have been also developed and are promising for use in the food industry due to the greater resistance of oil to rancidity [6,8,9]. Linoleic acid (LIO, omega-6) and oleic acid (OLE, omega-9) are also important unsaturated fatty acids in flax seeds. There is an inverse relationship between LIO and LIN levels: high-LIN varieties have low LIO content and low-LIN varieties have high LIO content [7,10]. Differences in the OLE content between varieties are less pronounced and can be about 2-fold [7,10]. Varieties with higher OLE content are promising for the production of oxidation-stable edible oil, chemical feedstock, and biodiesel [8,9].

Desaturases are known to play a key role in the synthesis of flax fatty acids by introducing double bonds into the hydrocarbon chain. SAD (stearoyl-ACP desaturases) catalyze the conversion of stearic acid to oleic acid, FAD2 (fatty acid desaturases 2) catalyze the conversion of oleic acid to linoleic acid, and FAD3 (fatty acid desaturases 3) catalyze the conversion of linoleic acid to linolenic acid [11–17]. In flax, 25 genes of the *SAD* and *FAD* families were identified: *SAD2-1*, *SAD2-2*, *SAD3-1*, *SAD3-2*, *FAD2a-1*, *FAD2a-2*, *FAD2b-1*, *FAD2b-2*, *FAD2c-1*, *FAD2c-2*, *FAD2d-1*, *FAD2d-2*, *FAD2e-1*, *FAD2e-2*, *FAD2f-1*, *FAD2f-2*, *FAD2g-1*, *FAD2g-2*, *FAD2h*, *FAD3a*, *FAD3b*, *FAD3c-1*, *FAD3c-2*, *FAD3d-1*, and *FAD3d-2* [18,19]. Mutations in the *FAD3a* and *FAD3b* genes are known to result in reduced linolenic acid content in oil [7,16,17,20–24]. The association of *FAD3a* and *FAD3b* expression with LIN and LIO accumulation was reported [16,17,25]. However, no correlation was found between the expression of *SAD1*, *SAD2*, *FAD2a*, *FAD2b*, *FAD3a*, and *FAD3b* and the FA composition of linseed oil in genotypes with the same desaturase isoforms but different FA composition [26]. Several studies investigated changes in expression levels of *FAD* and *SAD* genes during flax seed development and identified the stages at which these genes are most active: 20 days after flowering (DAF) for *FAD3* family genes [17]; 16, 22, and 30 DAF for genes of *SAD*, *FAD2*, and *FAD3* families with variation among genotypes [25]; 20 and 24 DAF for genes of *SAD*, *FAD2*, and *FAD3* families [26]; 15 and 20 DAF for genes of *FAD2* family [8]. The influence of growing conditions on linseed oil content and its FA composition is known, and the influence of temperature and humidity was particularly noted [10,27–30]. The above works contributed significantly to the understanding of the effects of genotype and environment on the expression of key FA synthesis genes during flax seed development. However, a holistic picture of how growing conditions affect the expression of *FAD* and *SAD* genes during seed development in different flax genotypes is lacking. It is also necessary to understand which genes from the *SAD*, *FAD2*, and *FAD3* families have the highest expression levels in seeds and, therefore, could contribute most to linseed oil FA synthesis at specific stages of seed development in different genotypes. These questions were the focus of our work.

2. Results

2.1. Results of Transcriptome Sequencing of Flax Seeds

Sequencing of 266 flax seed transcriptome libraries was performed. Ten flax varieties, which differed in the FA composition of oil and the presence of mutations in the *FAD3* genes leading to reduced LIN content, were used in the work (Table 1): high-LIN AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh; mid-LIN Raciol and AGT 422; low-LIN AGT 981, AGT 1535, and Lola. Seeds were collected at the 3, 7, 14, 21, and 28 day after flowering (DAF) from plants grown under conditions of 16 °C and overwatering (hereafter referred to as 16 °C), 20 °C and optimal watering (hereafter referred to as 20 °C), 24 °C and underwatering (hereafter referred as 24 °C). No data were obtained for variety AGT 422 at the 21 and 28 DAF at 24 °C and variety Lola at the 28 DAF at 24 °C, which is because of the negative impact of elevated temperature and insufficient watering on these genotypes. Sequencing was performed in two biological replicates for samples collected at the 3, 7, 14, and 21 DAF, and in one biological replicate for samples collected at the 28 DAF. On average, 2 million paired-end reads were obtained for each transcriptome library.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 December 2023 doi:10.20944/preprints202312.0947.v1

Table 1. Characteristics of the flax varieties used in this work.

Variety	OLE, %	LIO, %	LIN, %	*FAD3a* mutation G to A (Lu7.:16092348)	*FAD3b* mutation C to T (Lu12:1035655)	*FAD3a* mutation C to T (Lu7:16090340)
AGT 427	13.1	13.4	64.3	-	-	-
Atalante	16.4	14.7	58.2	-	-	-
Entre-Rios	20.1	16.5	52.5	-	-	-
Norlin	22.2	14.5	54.3	-	-	-
Pechersky kryazh	31.8	10.2	52.0	-	-	-
Raciol	15.5	39.2	35.0	-	+	-
AGT 422	18.3	35.5	32.6	-	+	-
AGT 981	18.5	67.2	3.2	+	+	-
AGT 1535	18.8	64.0	4.9	+	+	-
Lola	12.9	68.0	9.4	-	-	+

Note: The coordinates of the mutation sites are given according to the *L. usitatissimum* genome assembly GCA_000224295.2/ASM22429v2. Data on the FA composition and mutations are taken from the studies [7,10].

To visualize the relatedness of the studied samples, an MDS plot was constructed based on the expression of all identified transcripts (Figure 1). The samples were grouped mainly by developmental stage (i.e., DAF) and growth conditions (16 °C, 20 °C, and 24 °C) rather than belonging to a specific genotype.

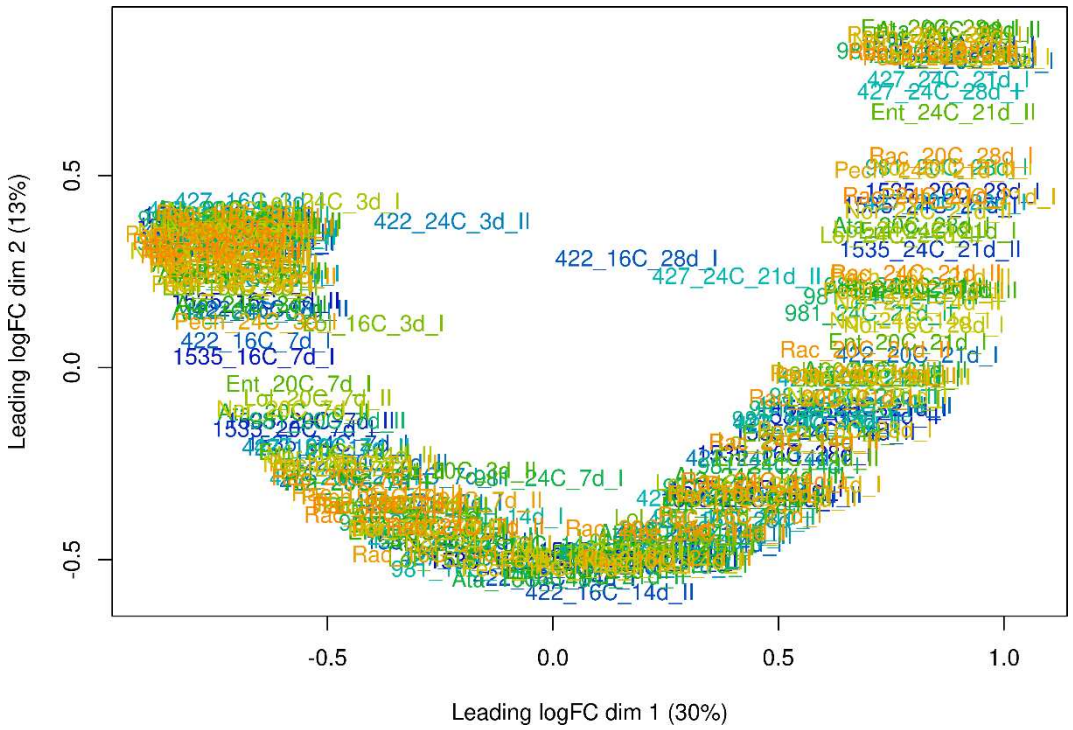

Figure 1. Multidimensional scaling plot (dimensions 1 and 2) for gene expression profiles in seeds (3, 7, 14, 21, and 28 DAF) of flax plants of 10 varieties (AGT 422 (422), AGT 427 (427), AGT 981 (981), AGT 1535 (1535), Atalante (Ata), Entre-Rios (Ent), Lola (Lol), Norlin (Nor), Pechersky kryazh (Pech), and Raciol (Rac)) grown under different temperature and watering conditions (16 °C, 20 °C, and 24 °C).

 doi:10.20944/preprints202312.0947.v1

The following abbreviations were used in the sample names: Variety_growth conditions_DAF_replicate.

2.2. Expression of the FAD and SAD Genes in Flax Seeds

Expression of genes of *FAD* and *SAD* families, which are known to play an important role in linseed FA synthesis [14,16,18,19,21,24,31,32], was evaluated. From 25 flax genes of *SAD* and *FAD* families (*SAD2-1*, *SAD2-2*, *SAD3-1*, *SAD3-2*, *FAD2a-1*, *FAD2a-2*, *FAD2b-1*, *FAD2b-2*, *FAD2c-1*, *FAD2c-2*, *FAD2d-1*, *FAD2d-2*, *FAD2e-1*, *FAD2e-2*, *FAD2f-1*, *FAD2f-2*, *FAD2g-1*, *FAD2g-2*, *FAD2h*, *FAD3a*, *FAD3b*, *FAD3c-1*, *FAD3c-2*, *FAD3d-1*, and *FAD3d-2*) [18,19,24], the highest expression levels in seeds were for *FAD3b*, *FAD3a*, *FAD2b-2*, *SAD3-1*, *SAD2-1*, *SAD2-2*, *SAD3-2*, *FAD2a-1*, and *FAD2a-2* (Supplementary Table S1). It can be assumed that these genes play the most important role in linseed oil synthesis among the *FAD* and *SAD* genes. For these genes, a more detailed analysis of expression profiles in different flax varieties grown at 16 °C, 20 °C, and 24 °C was performed.

2.3. Expression Profiles of the FAD3a and FAD3b Genes during Flax Seed Development under Different Temperature and Watering Conditions

FAD3 genes are known to play a key role in the conversion of LIO to LIN in linseed [16,17]. Among the *FAD3* genes, expression of *FAD3a* and *FAD3b* was the highest in the studied samples, ten times higher than that of *FAD3c-1*, *FAD3c-2*, *FAD3d-1*, and *FAD3d-2*. In different flax varieties, the expression of *FAD3a* and *FAD3b* generally changed similarly during seed development under the same growth conditions (Figures 2 and 3 respectively). Expression was minimal at the 3 DAF. The maximum expression of *FAD3a* and *FAD3b* genes at 16 °C was observed at the 21 or 28 DAF in most varieties. At 24 °C, the maximum expression of *FAD3a* and *FAD3b* genes was reached at the 14 DAF, after which a decrease was observed. At 20 °C, the maximum expression levels were observed at the 14 or 21 DAF, after which it decreased. It is worth noting that the maximum expression levels of *FAD3a* and *FAD3b* genes were similar at 16 °C, 20 °C, and 24 °C. Thus, increased temperature resulted in a shift of the maximum level of gene expression to earlier stages of seed development, while decreased temperature resulted in a shift to later stages of seed development.

Expression profiles of the *FAD3a* and *FAD3b* genes were very similar for the same variety under the same conditions (16 °C, 20 °C, and 24 °C). However, in varieties AGT 981, AGT 1535, and Lola, the level of *FAD3a* transcript was significantly lower than in the other varieties, while the level of *FAD3b* transcript was similar to the other varieties. Varieties AGT 981, AGT 1535, and Lola are known to have mutations in the *FAD3a* gene resulting in the stop codons and reduced LIN content [7]. Thus, the *FAD3a* transcript level was reduced in varieties with nonsense mutations of this gene. However, the situation was different for the *FAD3b* gene. Varieties AGT 422, AGT 981, AGT 1535, and Raciol, which carry the *FAD3b* mutation leading to the histidine-to-tyrosine substitution and LIN content reduction [7], had approximately the same expression level of this gene as the varieties without the mutation. It is worth noting that the expression level of *FAD3b* was slightly higher than that of *FAD3a* in all the varieties studied.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 December 2023 doi:10.20944/preprints202312.0947.v1

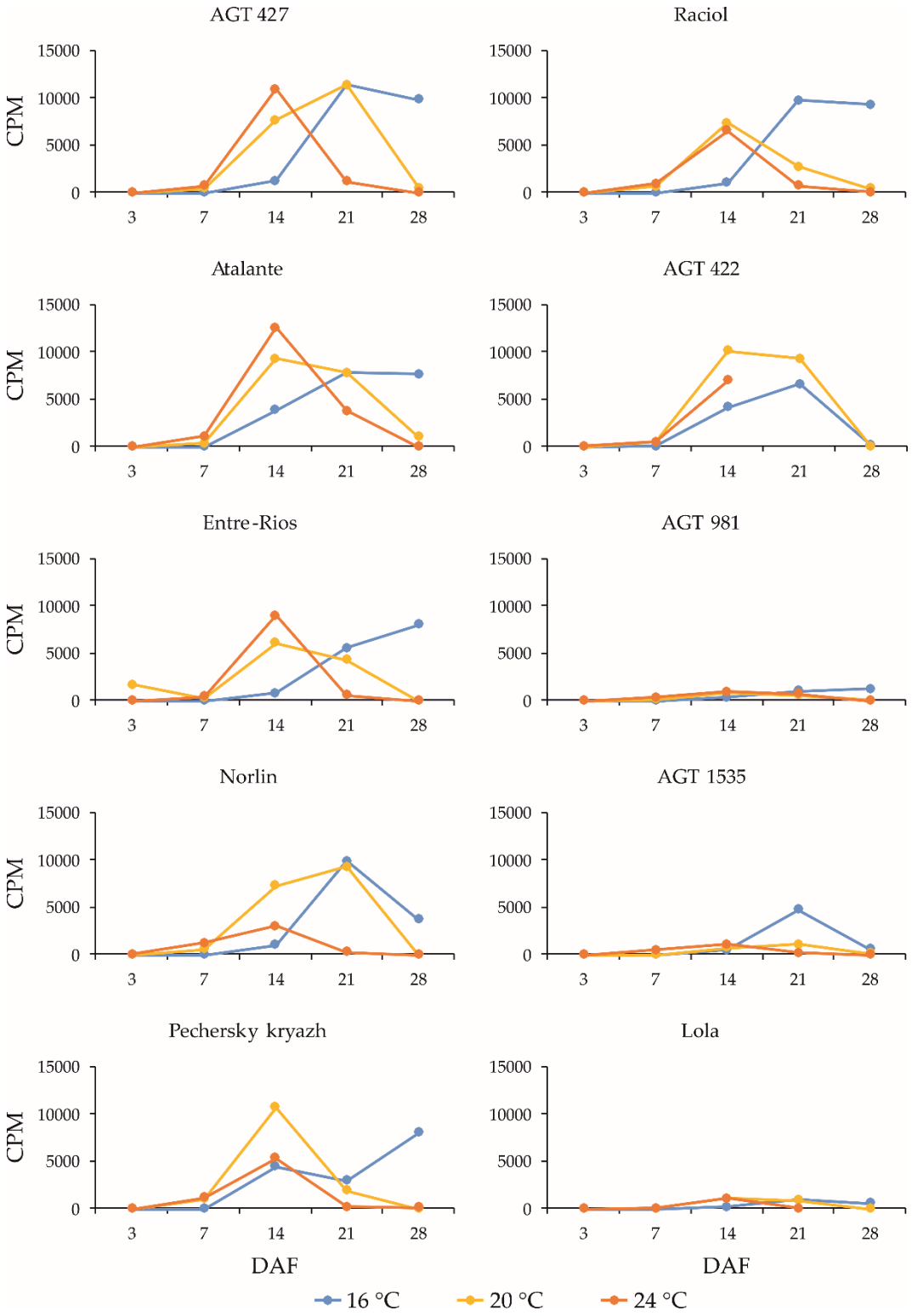

Figure 2. Expression profiles (3, 7, 14, 21, and 28 DAF) of the *FAD3a* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C). Data were missing for the 21 and 28 DAF at 24 °C for AGT 422 and the 28 DAF at 24 °C for Lola.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 December 2023 doi:10.20944/preprints202312.0947.v1

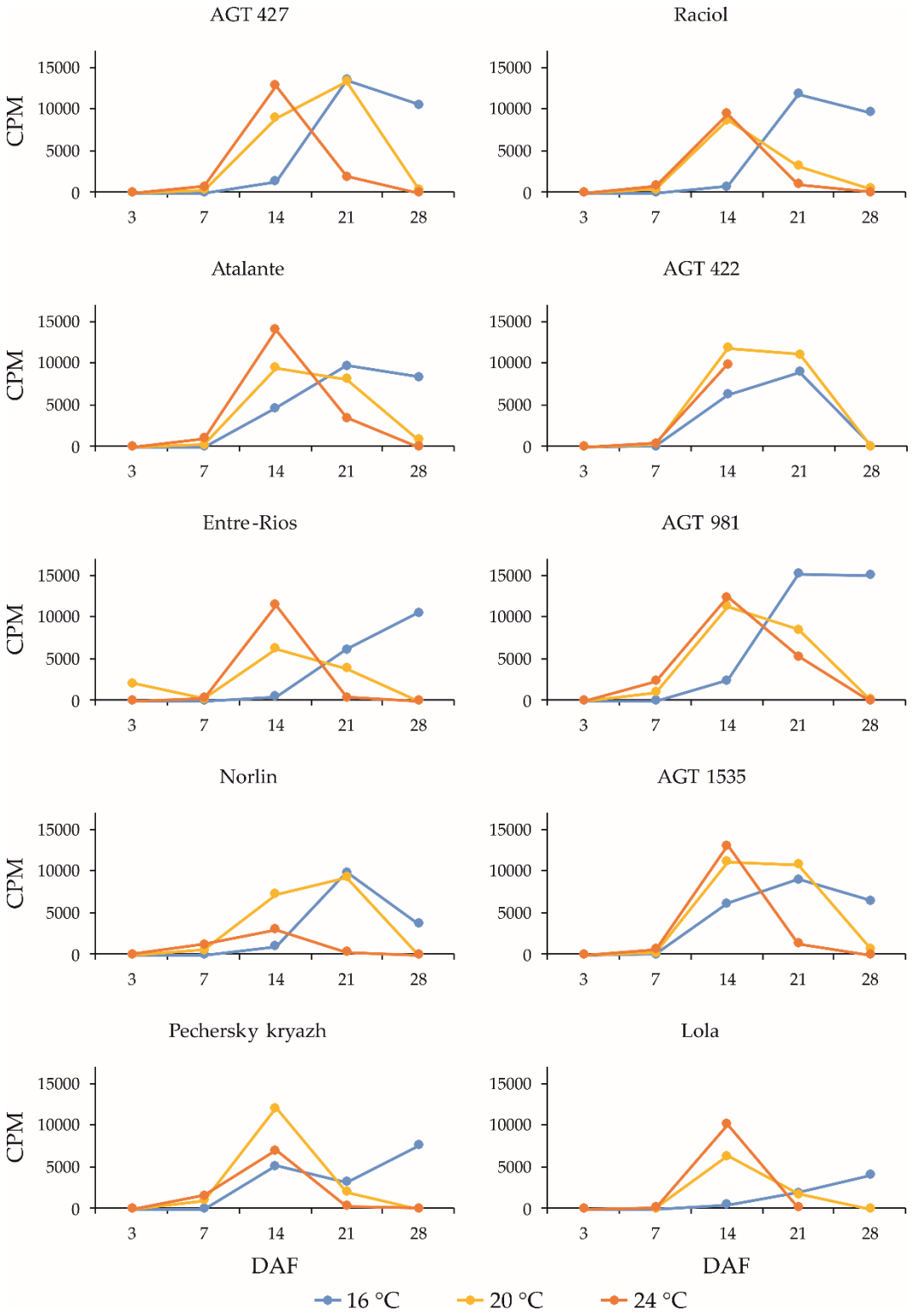

Figure 3. Expression profiles (3, 7, 14, 21, and 28 DAF) of the *FAD3b* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C). Data were missing for the 21 and 28 DAF at 24 °C for AGT 422 and the 28 DAF at 24 °C for Lola.

 doi:10.20944/preprints202312.0947.v1

2.4. Expression Profiles of the FAD2b-2, FAD2a-1, and FAD2a-2 Genes during Flax Seed Development under Different Temperature and Watering Conditions

FAD2 genes play an important role in the synthesis of linoleic acid from oleic acid [14,15]. Among the *FAD2* genes, the highest expression levels in flax seeds were found for *FAD2b-2*, *FAD2a-1*, and *FAD2a-2* (Supplementary Table S1). The expression level of *FAD2b-2* was several times higher than that of *FAD2a-1* and *FAD2a-2* in all varieties studied. The expression level of *FAD2a-1* was slightly higher than that of *FAD2a-2*. It should be noted that the *FAD2a-1* and *FAD2a-2* genes have very similar sequences, which differ by only 7 SNPs, which could introduce some bias in the estimation of their individual expression patterns [19]. For *FAD2b-2* (Figure 4), *FAD2a-1* (Supplementary Figure S1), and *FAD2a-2* (Supplementary Figure S2), as for *FAD3a* and *FAD3b*, cultivation at 16 °C resulted in altered expression profiles compared to 20 °C and 24 °C and a shift of the maximum expression levels to later stages of seed development (the 21 or 28 DAF). Expression profiles of *FAD2a-1* and *FAD2a-2* were very similar for the same variety under the same conditions, but slightly different from those of *FAD2b-2*.

All varieties showed drastic changes in expression levels of *FAD2b-2*, *FAD2a-1*, and *FAD2a-2* during seed development. At the 3 DAF, *FAD2b-2*, *FAD2a-1*, and *FAD2a-2* were barely expressed, but at the 14 DAF (at 20 °C and 24 °C) or later (at 16 °C), a dramatic increase in expression levels was observed. At the 28 DAF, there was a strong decrease in expression levels of the genes for all varieties at 20 °C and 24 °C, and there were differences in expression changes (increase, decrease, or retention compared to the 21 DAF) among varieties at 16 °C. A decrease in the maximum expression level of *FAD2b-2* at 16 °C compared to 20 °C and/or 24 °C can also be observed for some varieties. Pechersky kryazh had the highest OLE content (31.8%) among the 10 examined flax varieties, which was 1.5-2.0 times higher than that in other varieties. In this variety, the expression level of *FAD2b-2* was slightly lower than that in the majority of varieties studied. Expression level of *FAD2b-2* was also slightly lower in Norlin, which had increased OLE content (22.2%), and Lola, which had decreased OLE content (12.9%). For *FAD2a-1* and *FAD2a-2*, the differences in expression levels between Pechersky kryazh and other varieties were not very noticeable. At the same time, expression profiles of *FAD2b-2*, *FAD2a-1*, and *FAD2a-2* in the variety Pechersky kryazh were slightly different from other varieties, and the maximum expression levels were shifted to earlier stages of seed development (this was clearly visible for *FAD2a-1* and *FAD2a-2* at 20 °C and for *FAD2b-2* at 24 °C). Thus, the high OLE content in the variety Pechersky kryazh is unlikely related to the maximum *FAD2* expression levels. However, the shift of the maximum expression levels of *FAD2* genes to earlier stages of seed development could play a role in increasing the OLE content in linseed oil.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 December 2023 doi:10.20944/preprints202312.0947.v1

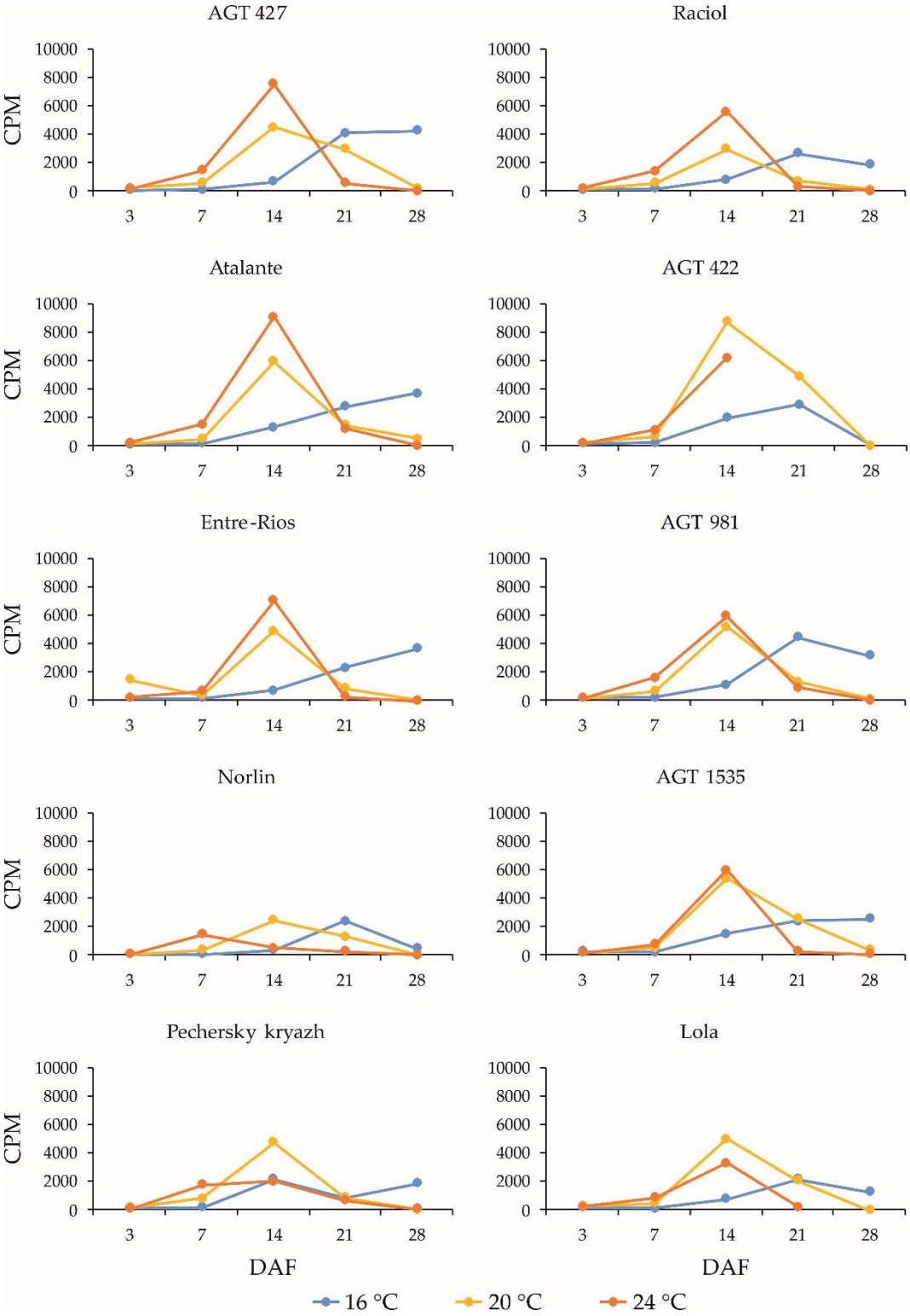

Figure 4. Expression profiles (3, 7, 14, 21, and 28 DAF) of the *FAD2b-2* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C). Data were missing for the 21 and 28 DAF at 24 °C for AGT 422 and the 28 DAF at 24 °C for Lola.

 doi:10.20944/preprints202312.0947.v1

2.5. Expression Levels of the SAD Genes during Flax Seed Development under Different Temperature and Watering Conditions

SAD genes are involved in the conversion of stearic acid to oleic acid in flax seeds [11–13]. In flax, all four identified *SAD* genes (*SAD2-1*, *SAD2-2*, *SAD3-1*, and *SAD3-2*) were rather highly expressed (Supplementary Table S1), with the highest level for *SAD3-1*. Expression profiles of *SAD2-1* (Figure 5) and *SAD2-2* (Supplementary Figure S3) for the same variety under the same conditions were very similar. The same was true for *SAD3-1* (Figure 6) and *SAD3-2* (Supplementary Figure S4). However, some differences in expression profiles were observed between the *SAD2-1*/*SAD2-2* and *SAD3-1*/*SAD3-2* pairs for the same samples, although the overall trend in expression changes was similar. During the seed development, an increase in expression levels of *SAD* genes was initially observed under all studied conditions. Then, at 20 °C and 24 °C, a decrease in expression levels was observed at the 21 or 28 DAF, while at 16 °C, different varieties showed an increase, decrease, or retention of expression levels. At the same time, at 20 °C and 24 °C for *SAD3-1* and *SAD3-2*, the maximum expression levels were reached at earlier developmental stages (the 14 DAF for most varieties) compared to *SAD2-1* and *SAD2-2* (the 21 DAF at 20 °C and a shift from the 14 to the 21 DAF at 24 °C for most varieties).

It can be noted that for *SAD2-1*/*SAD2-2* some expression was observed even at the 3 DAF, in contrast to the *SAD3-1*/*SAD3-2* and *FAD* genes, for which expression was practically absent at this stage. It is also interesting that expression changes during seed development (most notably at 20 °C) were more similar between *SAD3-1*/*SAD3-2* and *FAD2b-2* (reaching maximum levels at earlier stages) and between *SAD2-1*/*SAD2-2* and *FAD2a-1*/*FAD2a-2*, *FAD3a*, and *FAD3b* (reaching maximum levels at later stages). In Pechersky kryazh with high OLE content in the oil, characteristics similar to those described above for the *FAD2* genes were observed for the *SAD* genes. Thus, expression levels of *SAD* genes in the high-OLE variety Pechersky kryazh were close to those in the low-OLE variety Lola. However, in Pechersky kryazh, the *SAD* expression profiles were slightly different from other varieties, and the maximum expression levels were shifted to earlier stages of seed development (this was particularly noticeable for *SAD2-1* and *SAD2-2* at 20 °C and for *SAD3-1* and *SAD3-2* at 24 °C). Thus, the high OLE content in the variety Pechersky kryazh could be related not to its differences from other varieties in the maximum expression levels of *SAD* and *FAD2* genes, but to the shift of the maximum expression levels of these genes to earlier stages of seed development.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 December 2023 doi:10.20944/preprints202312.0947.v1

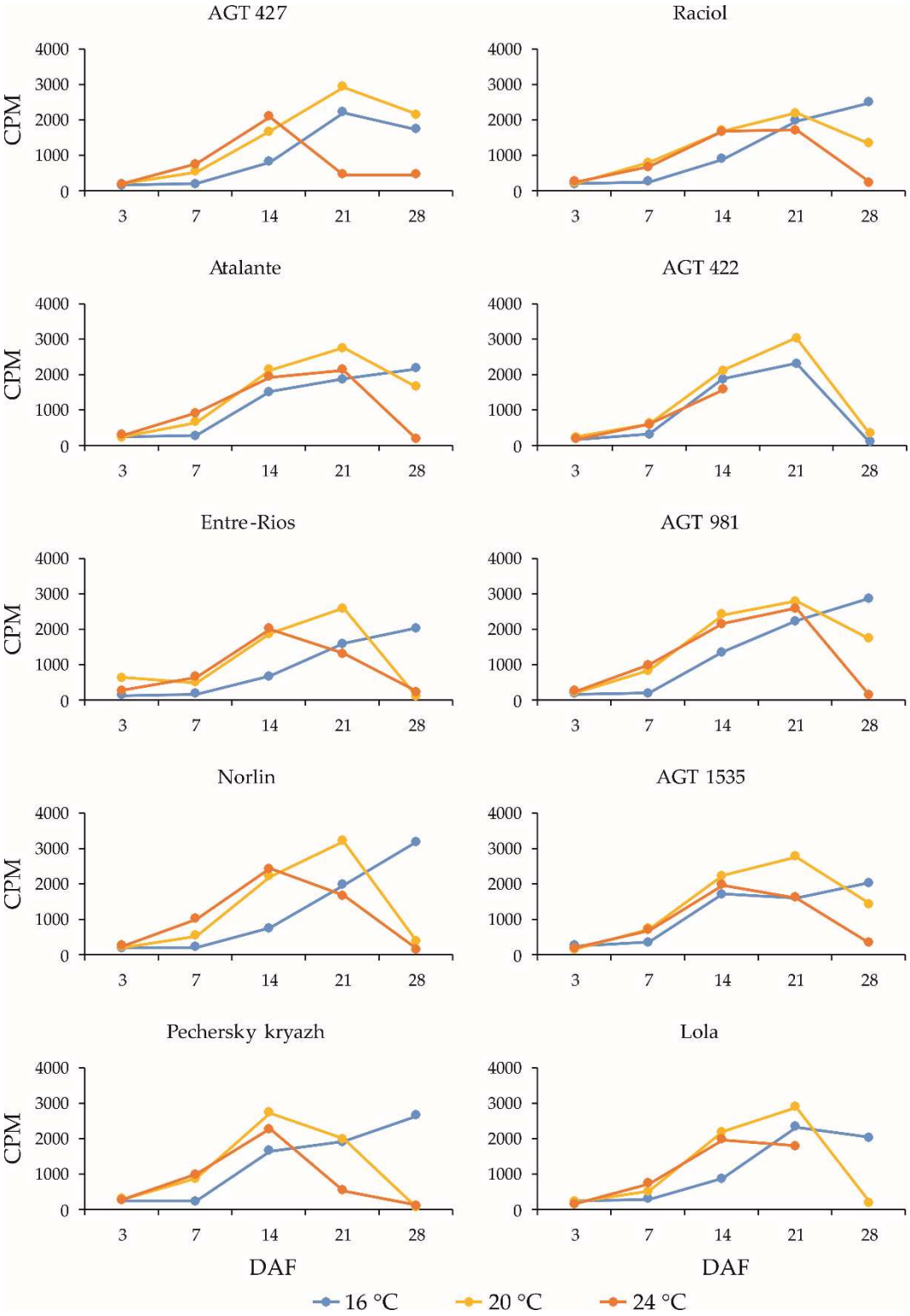

Figure 5. Expression profiles (3, 7, 14, 21, and 28 DAF) of the *SAD2-1* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C). Data were missing for the 21 and 28 DAF at 24 °C for AGT 422 and the 28 DAF at 24 °C for Lola.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 December 2023 doi:10.20944/preprints202312.0947.v1

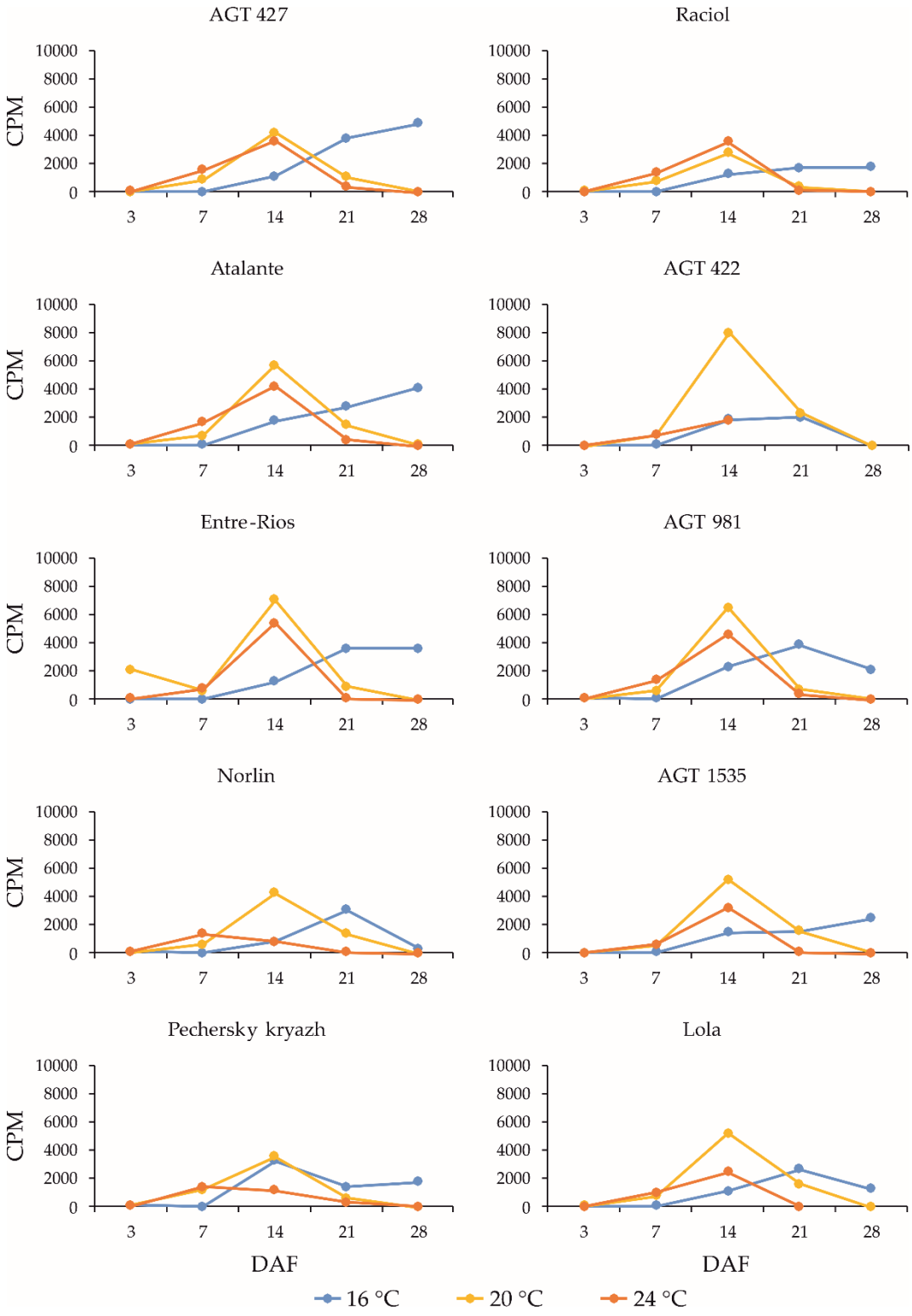

Figure 6. Expression profiles (3, 7, 14, 21, and 28 DAF) of the *SAD3-1* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C). Data were missing for the 21 and 28 DAF at 24 °C for AGT 422 and the 28 DAF at 24 °C for Lola.

 doi:10.20944/preprints202312.0947.v1

3. Discussion

It is known that the key role in the synthesis of unsaturated fatty acids in linseed oil is played by genes of *SAD*, *FAD2*, and *FAD3* families [11–17]. We analyzed the expression of these genes during seed development in a representative set of 10 flax varieties with different oil FA compositions grown under three temperature and watering conditions. In contrast to most previous works evaluating *SAD* and *FAD* gene expression in flax [8,16–18,25,26], we obtained individual expression data for all 25 known *SAD* and *FAD* genes, rather than expression levels just for a few genes from each family or common expression patterns for pairs of homologous genes. Due to the large number of works devoted to transcriptome analysis of different flax organs and tissues [33–44], we were previously able to determine which of the 25 *SAD* and *FAD* genes are expressed at high levels in flax seeds, and to estimate expression levels of these genes in other organs and tissues [19]. However, in that work, due to the lack of transcriptomic data, we were unable to assess the dynamics of expression of these genes during seed development, which was done in the present work for a representative set of flax varieties grown under different conditions affecting the FA composition of linseed oil.

The highest expression levels were detected for *FAD3b*, *FAD3a*, *FAD2b-2*, *SAD3-1*, *SAD2-1*, *SAD2-2*, *SAD3-2*, *FAD2a-1*, and *FAD2a-2*. In addition, expression levels of these genes changed tens and hundreds of times during seed development. In the early stages of development, expression of these genes was minimal, then a drastic increase was observed, but the dynamics of growth depended significantly on the growing conditions. The strongest increase was observed at 20 °C and 24 °C, with a maximum reached at the 14 or 21 DAF in most varieties, followed by a drastic decrease. At 24 °C compared to 20 °C, a shift of the maximum expression levels to earlier developmental stages was observed for the majority of highly expressed *FAD* and *SAD* genes. At 16 °C, the maximum expression levels of the analyzed genes were observed at later stages of seed development (predominantly at the 21 and 28 DAF), and the differences between varieties were quite pronounced. It is likely that among the *FAD* and *SAD* families, it is *FAD3b*, *FAD3a*, *FAD2b-2*, *SAD3-1*, *SAD2-1*, *SAD2-2*, *SAD3-2*, *FAD2a-1*, and *FAD2a-2* genes with high maximum expression levels and drastic expression changes during seed development that play a key role in the FA synthesis of linseed oil.

It is also possible to hypothesize how variations in *FAD* and *SAD* expression are reflected in the composition of linseed oil when plants are grown under different temperature and humidity conditions. Wet and cold summers are known to increase LIN+LIO level in linseed oil, whereas hot and dry summers are known to increase OLE content [10]. In our study, the decrease in *FAD2a-1* and *FAD2a-2* expression at higher temperature (24 °C) occurred at earlier stages of seed development compared to 20 °C and especially 16 °C. This trend was less clear for *FAD2b-2*, but it was also present in some genotypes. Probably under higher temperatures and insufficient watering, the *FAD2* genes do not have enough time to desaturate oleic acid to linoleic acid to the same extent as at lower temperatures, resulting in increased OLE and decreased LIO+LIN content. *FAD* genes are known to be involved in the response to a variety of stresses, including high and low temperatures [45]. The effects of increased and/or decreased temperatures on the expression of *FAD* genes were reported for banana [46], maize [47], *Gossypium* [48], cucumber [49], and some genotypes of soybean [50]. The effect of temperature on the production of LIN and LIO in olive was also shown [51]. For flax, we observed that temperature affects expression profiles of *FAD* and *SAD* genes, which could be reflected in the oil FA composition.

We found no correlation between expression levels of *FAD2* or *SAD* genes and OLE content. Thus, among the genotypes we studied, there was a high-oleic variety Pechersky kryazh, and expression levels of *FAD2* and *SAD* genes in it were close to those of the low-oleic variety Lola. At the same time, a shift of the maximum expression levels of *FAD* and *SAD* genes to earlier dates was observed in Pechersky kryazh compared to other varieties studied. This peculiarity could lead to a higher content of OLE in seeds of this variety. The strongest expression of *FAD* and *SAD* genes for this variety may occur during hotter summer periods than that for other varieties, which could further enhance the effect of increasing OLE content and decreasing LIO content. We grew flax plants under controlled conditions at constant temperatures, so differences in flowering time did not introduce a bias in the evaluation of expression levels. However, when plants are grown under field

conditions, the contribution of differences in flowering time between genotypes could also be reflected in the oil FA composition, because seed maturation may occur at different temperature/watering conditions.

Among the *SAD* genes, the highest expression level was found for *SAD3-1*. The expression level of its homolog *SAD3-2* was on average two times lower. Expression levels of homologous genes *SAD2-1* and *SAD2-2* were quite high, no more than 30% lower than that of *SAD3-1*. The expression level of *SAD2-1* was slightly higher than that of *SAD2-2*. It can be assumed that the *SAD3-1* gene plays the greatest role in the conversion of stearic acid to oleic acid in flax seeds, but *SAD2-1*, *SAD2-2*, and *SAD3-2* also contribute significantly to this process.

Among the *FAD2* genes, *FAD2b-2* had the highest expression level in most samples. However, the expression level of its homolog *FAD2b-1* was dozens of times lower. For the homologous *FAD2a-1* and *FAD2a-2*, expression levels were quite similar, with a slightly higher level of *FAD2a-1*. *FAD2b-2* is probably the key gene in the desaturation of oleic acid to linoleic acid in flax seeds, but *FAD2a-1* and *FAD2a-2* also contribute to this process. The role of *FAD2b-1* in linoleic acid synthesis in linseed oil is probably insignificant, which is also characteristic of the other *FAD2* genes studied, namely *FAD2c-1*, *FAD2c-2*, *FAD2d-1*, *FAD2d-2*, *FAD2e-1*, *FAD2e-2*, *FAD2f-1*, *FAD2f-2*, *FAD2g-1*, *FAD2g-2*, *FAD2h.*

Among the *FAD3* genes, *FAD3a* and *FAD3b* genes were characterized by the highest expression levels. Expression levels of these genes were similar, but in varieties AGT 981, AGT 1535, and Lola with nonsense mutations in the *FAD3a* gene leading to reduced LIN content, the level of *FAD3a* transcript was ten times lower than the level of *FAD3b* transcript. The low level of *FAD3a* transcript in the flax genotype with mutation of this gene leading to stop codon was previously reported and it was explained by nonsense-mediated mRNA decay [17]. No decrease in the *FAD3b* transcript level was detected in varieties with *FAD3b* missense mutation leading to the LIN content reduction. The fact that a missense mutation of the *FAD3b* gene does not lead to a change in its expression compared to other varieties was also reported earlier [17]. It was also shown previously that several *FAD3a* and *FAD3b* gene isoforms carrying mutations encode non-functional enzymes that are unable to convert LIO to LIN [24]. The contribution of *FAD3a* and *FAD3b* to the formation of LIN from LIO is probably close, but the expression level of *FAD3b* was slightly higher than that of *FAD3a*, so the role of *FAD3b* in the synthesis of linolenic acid could be slightly higher. This assumption is supported by the higher LIN content in flax varieties with mutations in the *FAD3a* gene compared to varieties with mutations in the *FAD3b* gene [7].

We observed similar expression profiles for *SAD3-1*, *SAD3-2*, and *FAD2b-2*. Expression profiles of *SAD2-1*, *SAD2-2*, *FAD2a-1*, *FAD2a-2*, *FAD3a*, and *FAD3b* were also similar. For the first group of genes, maximum expression levels were reached at earlier stages of seed development compared to the second group. There could be common regulatory mechanisms for the genes in each group, and these groups of genes could contribute somewhat differently to the synthesis of linseed oil at different stages of seed development.

Thus, we were able to identify *FAD* and *SAD* genes with the highest expression in flax seeds, which probably play an important role in the synthesis of OLE, LIO, and LIN of linseed oil. Expression profiles of the highly expressed *FAD* and *SAD* genes we examined were quite similar during seed development under the same conditions. All *FAD* and *SAD* genes showed dramatic changes in expression levels during seed development. The dependence of expression profiles on growth conditions was also observed: lower temperatures shifted the maximum expression levels to later stages of seed development, while higher temperatures often resulted in shifts of maximum expression levels to earlier stages or an earlier decrease of expression during seed development. These changes could be reflected in the FA composition of linseed oil. In addition, genotype-dependent features of *FAD* and *SAD* expression were found. A correlation between the transcript level and LIN content was found for the *FAD3a* gene, but for other *FAD* and *SAD* genes investigated, no pronounced associations between expression levels and OLE, LIO, or LIN content were found. Our work is important for understanding the role of *FAD* and *SAD* genes in linseed fatty acid synthesis and the contribution of genotype and environment to the expression of these genes. From

 doi:10.20944/preprints202312.0947.v1

an applied point of view, the results obtained can be used in marker-assisted selection and genome editing to create flax varieties with the desired fatty acid composition in seed, as well as in the development of recommendations for the optimal conditions of oilseed flax cultivation to obtain oil with the specific FA composition.

4. Materials and Methods

4.1. Flax Varieties

We used plants of 10 flax varieties: AGT 422, AGT 427, AGT 981, AGT 1535, Atalante, Entre-Rios, Lola, Norlin, Pechersky kryazh (namely, l. 1-2 from k-2889 according to the catalog of the Federal Research Center for Bast Fiber Crops, selection from Pechersky kryazh, originator N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR)), and Raciol. AGT 981, AGT 1535, and Lola had low LIN content; Raciol and AGT 422 had medium LIN content; AGT 427, Entre-Rios, Norlin, Atalante, and Pechersky kryazh had high LIN content (Table 1). Low and medium LIN levels were associated with mutations in the *FAD3a* and *FAD3b* genes [7], data are shown in Table 1.

4.2. Growth Conditions

Flax plants were grown in 15-liter pots with soil pretreated with fungicide. pH was maintained at about 5.5, the optimum level for flax. 32 seeds were sown per pot and three pots were planted for each variety. All plants were kept in the same conditions for one month after planting. The plants were then transferred to three climate chambers with different temperature regimes for growth. In the first chamber, the temperature was maintained at 16 °C, and watering was done every day. In the second chamber, the temperature was 20 °C, and watering was done every two days. In the third chamber, the temperature was 24 °C, and watering was done every three days.

4.3. Collection of Plant Material and RNA Isolation

The flower was marked with the date on the day it opened (day of flowering) and the data were recorded in a table. Seeds were collected at the 3, 7, 14, 21, and 28 day after flowering (DAF). For each variety, at least 10 samples were collected for each temperature/watering condition and each developmental stage analyzed. Seeds collected from individual capsules were placed in tubes and immediately frozen in liquid nitrogen. Plant material was placed in a low-temperature freezer at -70 °C.

For RNA isolation, seeds were first ground to a fine powder using disposable homogenization pestles (Helicon, Russia) inserted into a DeWalt DCD701D2 cordless drill (DeWalt, Germany) at a speed of 1200-1500 rpm in 1.5 mL tubes in liquid nitrogen without thawing the sample. RNA isolation was performed according to the protocol of Wang et al. [52] with some modifications. Briefly, 1 mL of pre-warmed to 65 °C CTAB lysis buffer (2% CTAB (neoFroxx GmbH, Germany), 2% PVP K30 (Pan-Reac AppliChem, Germany), 100mM Tris HCl pH 8.0 (Thermo Fisher Scientific, USA), 25mM EDTA (Thermo Fisher Scientific), 2 M NaCl (Scharlab, Spain), 2% β-mercaptoethanol (BioRad, USA)) was added to the homogenized sample. The homogenate was incubated in a TDB-120 thermostat (Biosan, Latvia) at 65 °C for 30 min with mixing every 10 min. Then, five identical samples (the same variety, growing condition, and developmental stage) were mixed as follows: 350 µl of homogenate was taken from each sample using a 1000 ml wide-bore pipette tip and added to a 2 ml tube. Next, 500 µl of homogenate was taken from the pool (the rest was frozen in liquid nitrogen) and 500 µl of CTAB lysis buffer pre-warmed to 65 °C was added. In the next step, an equal volume of cold chloroform (Acros Organics, USA) was added to the homogenate, vortexed for 30 sec, and then centrifuged on a microcentrifuge 5418R (Eppendorf, Germany) for 20 min at 10000 g and 4 °C. The aqueous phase was transferred to clean tubes, then an equal volume of cold chloroform was added, vortexed for 30 sec, and centrifuged for 10 min at 10000 g and 4 °C. The aqueous phase was transferred to clean tubes and 1/2 volume of 96% ethanol was added and mixed until homogeneous. Total RNA was then purified using the CleanRNA Standard Kit (Evrogen, Russia) according to the manufacturer's protocol with a DNAase I treatment step from the RNase-Free DNase Set (Qiagen, USA). RNA quality

 doi:10.20944/preprints202312.0947.v1

and concentration were evaluated using a gel electrophoresis, 2100 Bioanalyzer (Agilent Technologies, USA), and Qubit 4 fluorometer (Thermo Fisher Scientific). Only non-degraded RNA samples with a concentration of at least 20 ng/μL were used for further work.

4.4. Preparation and Sequencing of cDNA Libraries

Preparation of cDNA libraries for sequencing of flax seed transcriptomes was performed using the QIAseq Stranded mRNA Select Kit (Qiagen) according to the manufacturer's protocol. RNA from seeds of 10 flax varieties (AGT 422, AGT 427, AGT 981, AGT 1535, Atalante, Entre-Rios, Lola, Norlin, Pechersky kryazh, and Raciol) harvested at the 3, 7, 14, 21, and 28 DAF from plants grown under three different temperature/watering conditions were used. For the 3, 7, 14, and 21 DAF, cDNA libraries were prepared in 2 replicates, and for the 28 DAF – in 1 replicate (because of the difficulty of obtaining RNA of high quality and sufficient quantity for seeds of late developmental stages). The quality of the obtained cDNA libraries (agreement of the length of the obtained libraries with the expected ones and absence of adapter dimers) was evaluated on a 2100 Bioanalyzer (Agilent Technologies), and the concentration was evaluated on a Qubit 4 fluorometer (Thermo Fisher Scientific).

The resulting cDNA libraries (266 libraries in total, as the libraries for the variety AGT 422 at the 21 and 28 DAF at 24 °C and the variety Lola at the 28 DAF at 24 °C were excluded from the analysis due to low quality) were mixed equimolarly and sequenced on a NextSeq 2000 instrument (Illumina, USA) using the NextSeq 2000 P3 Reagents (100 Cycles) kit (Illumina) in 51 + 51 nucleotide format.

4.5. Expression Analysis

Raw reads were processed using Trimmomatic 0.38 [53]. The data was checked for the presence of adapter sequences, trimmed by quality (TRAILING:24 SLIDINGWINDOW:4:14), and filtered by length (MINLEN:40). Then, the PPline tool was used for expression analysis [54]. Transcriptomic reads were aligned to the variety Atlant genome (GCA_014858635.1 in NCBI Genome) [55], and counts per million (CPM) values were calculated for 25 genes: *SAD2-1* (Atlant transcript H1233_031351), *SAD2-2* (H1233_038408), *SAD3-1* (H1233_054424), *SAD3-2* (H1233_039970), *FAD2a-1* (H1233_058938), *FAD2a-2* (H1233_061927), *FAD2b-1* (H1233_075799), *FAD2b-2* (H1233_078572), *FAD2c-1* (H1233_075801), *FAD2c-2* (H1233_078571), *FAD2d-1* (H1233_041596), *FAD2d-2* (H1233_078570), *FAD2e-1* (H1233_075803), *FAD2e-2* (H1233_078569), *FAD2f-1* (H1233_075804), *FAD2f-2* (H1233_078567), *FAD2g-1* (H1233_075805), *FAD2g-2* (H1233_078566), *FAD2h* (H1233_078565), *FAD3a* (H1233_027729), *FAD3b* (H1233_038272), *FAD3c-1* (H1233_054813), *FAD3c-2* (H1233_039845), *FAD3d-1* (H1233_041596), and *FAD3d-2* (H1233_041890).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: Expression profiles (3, 7, 14, 21, and 28 DAF) of the *FAD2a-1* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C); Figure S2: Expression profiles (3, 7, 14, 21, and 28 DAF) of the *FAD2a-2* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C); Figure S3: Expression profiles (3, 7, 14, 21, and 28 DAF) of the *SAD2-2* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C); Figure S4: Expression profiles (3, 7, 14, 21, and 28 DAF) of the *SAD3-2* gene in high-LIN (AGT 427, Atalante, Entre-Rios, Norlin, and Pechersky kryazh), mid-LIN (Raciol and AGT 422), and low-LIN (AGT 981, AGT 1535, and Lola) flax varieties grown at 16 °C and overwatered (16 °C), 20 °C and optimal watered (20 °C), 24 °C and underwatered (24 °C); Table S1: Expression levels of *SAD* (*SAD2-1*, *SAD2-2*, *SAD3-1*, and *SAD3-2*), *FAD2* (*FAD2a-1*, *FAD2a-2*, *FAD2b-1*, *FAD2b-2*, *FAD2c-1*, *FAD2c-2*, *FAD2d-1*, *FAD2d-2*, *FAD2e-1*, *FAD2e-2*, *FAD2f-1*, *FAD2f-2*, and *FAD2g-2*), and *FAD3* (*FAD3a*, *FAD3b*, *FAD3c-1*, *FAD3c-2*, *FAD3d-1*, and *FAD3d-2*) genes in seeds (3, 7, 14, 21, and 28 DAF) of flax plants of 10 varieties (AGT 422 (422), AGT 427 (427), AGT 981 (981), AGT 1535 (1535), Atalante (Ata), Entre-

Rios (Ent), Lola (Lol), Norlin (Nor), Pechersky kryazh (Pech), and Raciol (Rac)) grown under different temperature and watering conditions (16 °C, 20 °C, and 24 °C).

Author Contributions: Conceptualization, T.A.R., A.A.D., and N.V.M.; performing experiments, E.N.P., L.V.P., R.O.N., T.A.R., A.A.G., D.A.Z., E.V.B., A.A.T., A.G.Y., and N.L.B.; data analysis, E.M.D., E.A.S., A.A.D., and N.V.M.; writing, E.N.P., E.M.D., A.A.D., and N.V.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the Russian Science Foundation, grant 21-16-00111.

Data Availability Statement: The raw sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1039849.

Acknowledgments: We thank the Center for Precision Genome Editing and Genetic Technologies for Biomedicine, EIMB RAS for providing the computing power and techniques for the data analysis. This work was performed using the equipment of EIMB RAS "Genome" center (http://www.eimb.ru/ru1/ckp/ccu_genome_ce.php).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Parikh, M.; Maddaford, T.G.; Austria, J.A.; Aliani, M.; Netticadan, T.; Pierce, G.N. Dietary Flaxseed as a Strategy for Improving Human Health. *Nutrients* **2019**, *11*, doi:10.3390/nu11051171.
2. Saini, R.K.; Prasad, P.; Sreedhar, R.V.; Akhilender Naidu, K.; Shang, X.; Keum, Y.S. Omega-3 Polyunsaturated Fatty Acids (PUFAs): Emerging Plant and Microbial Sources, Oxidative Stability, Bioavailability, and Health Benefits-A Review. *Antioxidants* **2021**, *10*, doi:10.3390/antiox10101627.
3. Al-Madhagy, S.; Ashmawy, N.S.; Mamdouh, A.; Eldahshan, O.A.; Farag, M.A. A comprehensive review of the health benefits of flaxseed oil in relation to its chemical composition and comparison with other omega-3-rich oils. *European journal of medical research* **2023**, *28*, 240, doi:10.1186/s40001-023-01203-6.
4. Campos, J.R.; Severino, P.; Ferreira, C.S.; Zielinska, A.; Santini, A.; Souto, S.B.; Souto, E.B. Linseed Essential Oil - Source of Lipids as Active Ingredients for Pharmaceuticals and Nutraceuticals. *Current medicinal chemistry* **2019**, *26*, 4537-4558, doi:10.2174/0929867325666181031105603.
5. Cloutier, S.; Ragupathy, R.; Niu, Z.; Duguid, S. SSR-based linkage map of flax (Linum usitatissimum L.) and mapping of QTLs underlying fatty acid composition traits. *Molecular Breeding* **2011**, *28*, 437-451, doi:10.1007/s11032-010-9494-1.
6. Muir, A.D.; Westcott, N.D. *Flax: The genus Linum*; CRC Press: 2003.
7. Dmitriev, A.A.; Kezimana, P.; Rozhmina, T.A.; Zhuchenko, A.A.; Povkhova, L.V.; Pushkova, E.N.; Novakovskiy, R.O.; Pavelek, M.; Vladimirov, G.N.; Nikolaev, E.N.; et al. Genetic diversity of SAD and FAD genes responsible for the fatty acid composition in flax cultivars and lines. *BMC Plant Biology* **2020**, *20*, 301, doi:10.1186/s12870-020-02499-w.
8. Chen, Y.; Zhou, X.R.; Zhang, Z.J.; Dribnenki, P.; Singh, S.; Green, A. Development of high oleic oil crop platform in flax through RNAi-mediated multiple FAD2 gene silencing. *Plant cell reports* **2015**, *34*, 643-653, doi:10.1007/s00299-015-1737-5.
9. Brutch, N.; Porokhoviniva, E.; Shelenga, T. Perspectives of the creation of oil flax varieties for the specialized purpose. *Agrarian Reporter of South-East* **2016**, 1-2.
10. Porokhovinova, E.; Shelenga, T.; Kosykh, L.; Sanin, A.; Kazarina, A.; Kutuzova, S.; Pavlov, A.; Brutch, N. Biochemical diversity of fatty acid composition in flax from VIR genetic collection and effect of environment on its development. *Ecological genetics* **2016**, *14*, 13, doi:10.17816/ecogen14113-26.
11. Jain, R.K.; Thompson, R.G.; Taylor, D.C.; MacKenzie, S.L.; McHughen, A.; Rowland, G.G.; Tenaschuk, D.; Coffey, M. Isolation and characterization of two promoters from linseed for genetic engineering. *Crop science* **1999**, *39*, 1696-1701.
12. Singh, S.; McKinney, S.; Green, A. Sequence of a cDNA from Linum usitatissimum encoding the stearoyl-acyl carrier protein desaturase. *Plant physiology* **1994**, *104*, 1075, doi:10.1104/pp.104.3.1075.
13. Fofana, B.; Duguid, S.; Cloutier, S. Cloning of fatty acid biosynthetic genes β-ketoacyl CoA synthase, fatty acid elongase, stearoyl-ACP desaturase, and fatty acid desaturase and analysis of expression in the early developmental stages of flax (Linum usitatissimum L.) seeds. *Plant Science* **2004**, *166*, 1487-1496.

14. Khadake, R.M.; Ranjekar, P.K.; Harsulkar, A.M. Cloning of a novel omega-6 desaturase from flax (Linum usitatissimum L.) and its functional analysis in Saccharomyces cerevisiae. *Molecular biotechnology* **2009**, *42*, 168-174, doi:10.1007/s12033-009-9150-3.
15. Krasowska, A.; Dziadkowiec, D.; Polinceusz, A.; Plonka, A.; Łukaszewicz, M. Cloning of flax oleic fatty acid desaturase and its expression in yeast. *Journal of the American Oil Chemists' Society* **2007**, *84*, 809-816.
16. Vrinten, P.; Hu, Z.; Munchinsky, M.A.; Rowland, G.; Qiu, X. Two FAD3 desaturase genes control the level of linolenic acid in flax seed. *Plant physiology* **2005**, *139*, 79-87, doi:10.1104/pp.105.064451.
17. Banik, M.; Duguid, S.; Cloutier, S. Transcript profiling and gene characterization of three fatty acid desaturase genes in high, moderate, and low linolenic acid genotypes of flax (Linum usitatissimum L.) and their role in linolenic acid accumulation. *Genome* **2011**, *54*, 471-483, doi:10.1139/g11-013.
18. You, F.M.; Li, P.; Kumar, S.; Ragupathy, R.; Li, Z.; Fu, Y.-B.; Cloutier, S. Genome-wide identification and characterization of the gene families controlling fatty acid biosynthesis in flax (Linum usitatissimum L). *J Proteomics Bioinform* **2014**, *7*, 310-326.
19. Dvorianinova, E.M.; Zinovieva, O.L.; Pushkova, E.N.; Zhernova, D.A.; Rozhmina, T.A.; Povkhova, L.V.; Novakovskiy, R.O.; Sigova, E.A.; Turba, A.A.; Borkhert, E.V.; et al. Key FAD2, FAD3, and SAD Genes Involved in the Fatty Acid Synthesis in Flax Identified Based on Genomic and Transcriptomic Data. *International journal of molecular sciences* **2023**, *24*, doi:10.3390/ijms241914885.
20. Rajwade, A.V.; Joshi, R.S.; Kadoo, N.Y.; Gupta, V.S. Sequence characterization and in silico structure prediction of fatty acid desaturases in linseed varieties with differential fatty acid composition. *Journal of the science of food and agriculture* **2016**, *96*, 4896-4906, doi:10.1002/jsfa.7775.
21. Thambugala, D.; Duguid, S.; Loewen, E.; Rowland, G.; Booker, H.; You, F.M.; Cloutier, S. Genetic variation of six desaturase genes in flax and their impact on fatty acid composition. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik* **2013**, *126*, 2627-2641, doi:10.1007/s00122-013-2161-2.
22. Porokhovinova, E.A.; Shelenga, T.V.; Matveeva, T.V.; Pavlov, A.V.; Grigorieva, E.A.; Brutch, N.B. Polymorphism of genes controlling low level of linolenic acid in lines from VIR flax genetic collection. *Ecological genetics* **2019**, *17*, 5-19.
23. Povkhova, L.V.; Pushkova, E.N.; Rozhmina, T.A.; Zhuchenko, A.A.; Frykin, R.I.; Novakovskiy, R.O.; Dvorianinova, E.M.; Gryzunov, A.A.; Borkhert, E.V.; Sigova, E.A.; et al. Development and Complex Application of Methods for the Identification of Mutations in the FAD3A and FAD3B Genes Resulting in the Reduced Content of Linolenic Acid in Flax Oil. *Plants* **2022**, *12*, doi:10.3390/plants12010095.
24. Radovanovic, N.; Thambugala, D.; Duguid, S.; Loewen, E.; Cloutier, S. Functional characterization of flax fatty acid desaturase FAD2 and FAD3 isoforms expressed in yeast reveals a broad diversity in activity. *Molecular biotechnology* **2014**, *56*, 609-620, doi:10.1007/s12033-014-9737-1.
25. Rajwade, A.V.; Kadoo, N.Y.; Borikar, S.P.; Harsulkar, A.M.; Ghorpade, P.B.; Gupta, V.S. Differential transcriptional activity of SAD, FAD2 and FAD3 desaturase genes in developing seeds of linseed contributes to varietal variation in alpha-linolenic acid content. *Phytochemistry* **2014**, *98*, 41-53, doi:10.1016/j.phytochem.2013.12.002.
26. Thambugala, D.; Cloutier, S. Fatty acid composition and desaturase gene expression in flax (Linum usitatissimum L.). *Journal of applied genetics* **2014**, *55*, 423-432, doi:10.1007/s13353-014-0222-0.
27. Xie, D.; Dai, Z.; Yang, Z.; Tang, Q.; Deng, C.; Xu, Y.; Wang, J.; Chen, J.; Zhao, D.; Zhang, S.; et al. Combined genome-wide association analysis and transcriptome sequencing to identify candidate genes for flax seed fatty acid metabolism. *Plant science : an international journal of experimental plant biology* **2019**, *286*, 98-107, doi:10.1016/j.plantsci.2019.06.004.
28. You, F.M.; Xiao, J.; Li, P.; Yao, Z.; Jia, G.; He, L.; Kumar, S.; Soto-Cerda, B.; Duguid, S.D.; Booker, H.M.; et al. Genome-Wide Association Study and Selection Signatures Detect Genomic Regions Associated with Seed Yield and Oil Quality in Flax. *International journal of molecular sciences* **2018**, *19*, doi:10.3390/ijms19082303.
29. Marketta, S.; Juha-Matti, P.; Merja, E.; Ari, K.; Lauri, J.; Veli, H. Yield, SDG lignan, cadmium, lead, oil and protein contents of linseed (Linum usitatissimum L.) cultivated in trials and at different farm conditions in the south-western part of Finland. *Agricultural and Food Science* **2013**, *22*, doi:10.23986/afsci.7355.
30. Dybing, C.D.; Zimmerman, D.C. Fatty Acid accumulation in maturing flaxseeds as influenced by environment. *Plant physiology* **1966**, *41*, 1465-1470, doi:10.1104/pp.41.9.1465.

31. Fofana, B.; Cloutier, S.; Duguid, S.; Ching, J.; Rampitsch, C. Gene expression of stearoyl-ACP desaturase and delta12 fatty acid desaturase 2 is modulated during seed development of flax (Linum usitatissimum). *Lipids* **2006**, *41*, 705-712, doi:10.1007/s11745-006-5021-x.
32. Khadake, R.; Khonde, V.; Mhaske, V.; Ranjekar, P.; Harsulkar, A. Functional and bioinformatic characterisation of sequence variants of Fad3 gene from flax. *Journal of the science of food and agriculture* **2011**, *91*, 2689-2696, doi:10.1002/jsfa.4515.
33. Dash, P.K.; Rai, R.; Mahato, A.K.; Gaikwad, K.; Singh, N.K. Transcriptome Landscape at Different Developmental Stages of a Drought Tolerant Cultivar of Flax (Linum usitatissimum). *Frontiers in chemistry* **2017**, *5*, 82, doi:10.3389/fchem.2017.00082.
34. Dmitriev, A.A.; Kudryavtseva, A.V.; Krasnov, G.S.; Koroban, N.V.; Speranskaya, A.S.; Krinitsina, A.A.; Belenikin, M.S.; Snezhkina, A.V.; Sadritdinova, A.F.; Kishlyan, N.V.; et al. Gene expression profiling of flax (Linum usitatissimum L.) under edaphic stress. *BMC Plant Biol* **2016**, *16*, 237, doi:10.1186/s12870-016-0927-9.
35. Galindo-Gonzalez, L.; Deyholos, M.K. RNA-seq Transcriptome Response of Flax (Linum usitatissimum L.) to the Pathogenic Fungus Fusarium oxysporum f. sp. lini. *Frontiers in plant science* **2016**, *7*, 1766, doi:10.3389/fpls.2016.01766.
36. Dmitriev, A.A.; Krasnov, G.S.; Rozhmina, T.A.; Novakovskiy, R.O.; Snezhkina, A.V.; Fedorova, M.S.; Yurkevich, O.Y.; Muravenko, O.V.; Bolsheva, N.L.; Kudryavtseva, A.V.; et al. Differential gene expression in response to Fusarium oxysporum infection in resistant and susceptible genotypes of flax (Linum usitatissimum L.). *BMC Plant Biol* **2017**, *17*, 253, doi:10.1186/s12870-017-1192-2.
37. Dmitriev, A.A.; Novakovskiy, R.O.; Pushkova, E.N.; Rozhmina, T.A.; Zhuchenko, A.A.; Bolsheva, N.L.; Beniaminov, A.D.; Mitkevich, V.A.; Povkhova, L.V.; Dvorianinova, E.M.; et al. Transcriptomes of Different Tissues of Flax (Linum usitatissimum L.) Cultivars With Diverse Characteristics. *Frontiers in genetics* **2020**, *11*, 565146, doi:10.3389/fgene.2020.565146.
38. Dmitriev, A.A.; Krasnov, G.S.; Rozhmina, T.A.; Zyablitsin, A.V.; Snezhkina, A.V.; Fedorova, M.S.; Pushkova, E.N.; Kezimana, P.; Novakovskiy, R.O.; Povkhova, L.V.; et al. Flax (Linum usitatissimum L.) response to non-optimal soil acidity and zinc deficiency. *BMC Plant Biol* **2019**, *19*, 54, doi:10.1186/s12870-019-1641-1.
39. Petrova, A.; Kozlova, L.; Gorshkov, O.; Nazipova, A.; Ageeva, M.; Gorshkova, T. Cell Wall Layer Induced in Xylem Fibers of Flax Upon Gravistimulation Is Similar to Constitutively Formed Cell Walls of Bast Fibers. *Frontiers in plant science* **2021**, *12*, 660375, doi:10.3389/fpls.2021.660375.
40. Gorshkov, O.; Chernova, T.; Mokshina, N.; Gogoleva, N.; Suslov, D.; Tkachenko, A.; Gorshkova, T. Intrusive Growth of Phloem Fibers in Flax Stem: Integrated Analysis of miRNA and mRNA Expression Profiles. *Plants* **2019**, *8*, doi:10.3390/plants8020047.
41. Gorshkova, T.; Chernova, T.; Mokshina, N.; Gorshkov, V.; Kozlova, L.; Gorshkov, O. Transcriptome Analysis of Intrusively Growing Flax Fibers Isolated by Laser Microdissection. *Scientific reports* **2018**, *8*, 14570, doi:10.1038/s41598-018-32869-2.
42. Gorshkov, O.; Mokshina, N.; Ibragimova, N.; Ageeva, M.; Gogoleva, N.; Gorshkova, T. Phloem fibres as motors of gravitropic behaviour of flax plants: level of transcriptome. *Functional plant biology : FPB* **2018**, *45*, 203-214, doi:10.1071/FP16348.
43. Gorshkov, O.; Mokshina, N.; Gorshkov, V.; Chemikosova, S.; Gogolev, Y.; Gorshkova, T. Transcriptome portrait of cellulose-enriched flax fibres at advanced stage of specialization. *Plant molecular biology* **2017**, *93*, 431-449, doi:10.1007/s11103-016-0571-7.
44. Gao, P.; Qiu, S.; Ma, X.; Parkin, I.A.P.; Xiang, D.; Datla, R. Spatiotemporal Transcriptomic Atlas of Developing Embryos and Vegetative Tissues in Flax. *Plants* **2022**, *11*, doi:10.3390/plants11152031.
45. Xiao, R.; Zou, Y.; Guo, X.; Li, H.; Lu, H. Fatty acid desaturases (FADs) modulate multiple lipid metabolism pathways to improve plant resistance. *Molecular biology reports* **2022**, *49*, 9997-10011, doi:10.1007/s11033-022-07568-x.
46. Cheng, C.; Liu, F.; Sun, X.; Wang, B.; Liu, J.; Ni, X.; Hu, C.; Deng, G.; Tong, Z.; Zhang, Y.; et al. Genome-wide identification of FAD gene family and their contributions to the temperature stresses and mutualistic and parasitic fungi colonization responses in banana. *International journal of biological macromolecules* **2022**, *204*, 661-676, doi:10.1016/j.ijbiomac.2022.02.024.

47. Zhao, X.; Wei, J.; He, L.; Zhang, Y.; Zhao, Y.; Xu, X.; Wei, Y.; Ge, S.; Ding, D.; Liu, M.; et al. Identification of Fatty Acid Desaturases in Maize and Their Differential Responses to Low and High Temperature. *Genes* **2019**, *10*, doi:10.3390/genes10060445.
48. Yurchenko, O.P.; Park, S.; Ilut, D.C.; Inmon, J.J.; Millhollon, J.C.; Liechty, Z.; Page, J.T.; Jenks, M.A.; Chapman, K.D.; Udall, J.A.; et al. Genome-wide analysis of the omega-3 fatty acid desaturase gene family in Gossypium. *BMC Plant Biol* **2014**, *14*, 312, doi:10.1186/s12870-014-0312-5.
49. Dong, C.J.; Cao, N.; Zhang, Z.G.; Shang, Q.M. Characterization of the Fatty Acid Desaturase Genes in Cucumber: Structure, Phylogeny, and Expression Patterns. *PloS one* **2016**, *11*, e0149917, doi:10.1371/journal.pone.0149917.
50. Narayanan, S.; Zoong-Lwe, Z.S.; Gandhi, N.; Welti, R.; Fallen, B.; Smith, J.R.; Rustgi, S. Comparative Lipidomic Analysis Reveals Heat Stress Responses of Two Soybean Genotypes Differing in Temperature Sensitivity. *Plants* **2020**, *9*, doi:10.3390/plants9040457.
51. D'Angeli, S.; Altamura, M.M. Unsaturated Lipids Change in Olive Tree Drupe and Seed during Fruit Development and in Response to Cold-Stress and Acclimation. *International journal of molecular sciences* **2016**, *17*, doi:10.3390/ijms17111889.
52. Wang, L.; Stegemann, J.P. Extraction of high quality RNA from polysaccharide matrices using cetyltrimethylammonium bromide. *Biomaterials* **2010**, *31*, 1612-1618, doi:10.1016/j.biomaterials.2009.11.024.
53. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30*, 2114-2120, doi:10.1093/bioinformatics/btu170.
54. Krasnov, G.S.; Dmitriev, A.A.; Kudryavtseva, A.V.; Shargunov, A.V.; Karpov, D.S.; Uroshlev, L.A.; Melnikova, N.V.; Blinov, V.M.; Poverennaya, E.V.; Archakov, A.I.; et al. PPLine: An Automated Pipeline for SNP, SAP, and Splice Variant Detection in the Context of Proteogenomics. *Journal of proteome research* **2015**, *14*, 3729-3737, doi:10.1021/acs.jproteome.5b00490.
55. Dmitriev, A.A.; Pushkova, E.N.; Novakovskiy, R.O.; Beniaminov, A.D.; Rozhmina, T.A.; Zhuchenko, A.A.; Bolsheva, N.L.; Muravenko, O.V.; Povkhova, L.V.; Dvorianinova, E.M.; et al. Genome Sequencing of Fiber Flax Cultivar Atlant Using Oxford Nanopore and Illumina Platforms. *Frontiers in genetics* **2020**, *11*, 590282, doi:10.3389/fgene.2020.590282.