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Impact of Mutations in Soybean Oleate and Linoleate Desaturase Genes on Seed Germinability of Heat-Stressed Plants

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Abstract: Soybean is the primary oilseed crop in the United States, with significant industrial value. Understanding the molecular mechanisms of heat stress tolerance in soybean plants is critical for developing stress-resistant cultivars. Current knowledge about the role of fatty acid desaturases (FADs) in modulating membrane fluidity under abiotic stress prompted this investigation into the impact of mutations in the *FAD* genes on seed germination from heat-stressed plants. In soybean plants, exposure to heat stress during anthesis is known to significantly reduce seed germination. In silico expression analysis indicated high expression levels of the soybean *FAD2* and *FAD3* genes in the leaves. Therefore, a detailed expression analysis of these genes was conducted using qRT-PCR from leaf tissue. Generally, downregulation of these genes was observed in the mutants; however, two genes, *FAD3A* and *FAD2-3*, showed a more than 2-fold increase in expression in six out of ten mutants under heat stress. This upregulation was particularly pronounced (7-fold) in the mutant S17CR-170. Correlation analysis revealed a positive correlation (up to 0.48) between the expression level of *FAD3A*, *FAD3B*, *FAD3C*, and *FAD2-3* and the decline in germination from heat-stressed plants. This suggests these *FAD* genes may act as negative regulators of germination under heat stress conditions.

Keywords: soybean; heat stress; fatty acid desaturase mutants; expression analysis; germination

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1. Introduction

Soybean (*Glycine max* L., $2n = 40$) is an important legume crop that serves as a rich source of dietary oil and protein for human and animal consumption. It is also used as a raw material for various medical and industrial purposes [1]. Soybean is the leading oilseed crop in the U.S., accounting for about 90% of the total oilseed production [2]. Consequently, continuous efforts are being undertaken to develop soybean cultivars that are tolerant/resistant to adverse environmental conditions.

Heat and drought are two major abiotic stresses that significantly impact the soybean yield. The exponential increase in fossil fuel use since industrialization, along with other anthropogenic activities, has led to a notable rise in global temperatures, particularly during the summer crop growing season. This rise in temperature has caused erratic rainfall patterns during the growing season, posing a significant threat to global food security.

The U.S. led global soybean production until 2018, with its decline attributed partly to a global warming-induced decline in productivity and primarily to increased soybean acreage

in Brazil [3,4]. To mitigate heat-stress-triggered losses, the producers in the mid-Southern U.S. adopted an Early Soybean Production System (ESPS). Under this management strategy, early-maturing soybean varieties are planted in zones typically suitable for late-maturing varieties with the aim being to skip heat stress during anthesis by flowering early. This strategy, however effective, presents a unique challenge as plants sown under ESPS undergo seed maturation under heat stress [5].

Heat stress can occur at any developmental stage during soybean growth; however, it is most damaging during anthesis and seed development [6]. For instance, according to Lobel and Asner [7], an increase in temperature by 1 °C results in up to a 17% decline in soybean yield. Similarly, according to Schauberger et al. [8], soybean yield under rainfed conditions declines by up to 6% if the temperature rises above 30 °C during the growing season. Heat stress occurring at the time of anthesis or seed development causes a reduction in pollen viability, seed weight, and seed size. It also results in a loss of seed viability and vigor, leading to poor germination. Additionally, it causes seed coat wrinkling and discoloration, leading to poor physical appeal and reduced economic value [9–12].

Heat stress in crop plants generally results in several complex biochemical and physiological alterations that limit productivity [13]. Specifically, heat stress reduces assimilate production and translocation, increases carbohydrate starvation, and causes high respiration, especially under high nighttime temperatures [14]. Therefore, attempts are being made to understand the molecular mechanisms of heat stress tolerance in crop plants.

In soybean, molecular mechanisms of heat stress tolerance have been studied, and a few genes involved in the process have been identified. Similarly, several differentially expressed genes under heat stress have been identified in different tissues, including the leaf, pod, sepal, anther, and stigma [15], in seedlings [16], and at various developmental stages, such as leaves at the reproductive stage [17]. These early studies suggested that the molecular mechanisms of heat stress tolerance are complex and differ at various developmental stages, engaging different physiological and metabolic pathways. The physiological pathways impacted by heat stress in soybean include major pathways such as photosynthesis, stomatal conductance, and transpiration. While stomatal conductance and transpiration are reported to increase due to heat stress, photosynthesis is generally inhibited [17,18]. In another study, four different regimes of day and night temperatures were examined for their impacts on the physicochemical, agronomic, and genetic factors of two Chinese soybean genotypes. Physicochemical parameters included physiological traits like photosynthesis rate, stomatal conductance, and transpiration rate, as well as biochemical traits like superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) content. Agronomic traits included seeds and pods per plant and genetic factors included expression changes for the *GmDNJ1*, *GmDREB1*, *GmHSF-34*, *GmPYL21*, *GmPIF4b*, *GmPIP1;6*, *GmGBP1*, *GmHsp90A2*, *GmTIP2;6*, and *GmEF8* genes. The overall results revealed that a temperature regime of 35 °C during the day and 27 °C at night was the most suitable for growing Chinese soybean genotypes, due to the significant increase in agronomic trait values and gene expression changes observed under this temperature regimen [19]. This finding highlighted genotypic differences in how different soybean genotypes respond to heat stress.

Lipidomic, proteomic, and metabolomic studies were carried out to further understand the molecular mechanisms of heat tolerance [11,20,21]. For instance, a detailed study of leaf metabolites revealed the importance of sugar and nitrogen metabolism under heat and drought stress in soybean [22]. Similarly, temperature- and genotype-specific differences were observed in the seed metabolome, and a diverse set of antioxidant metabolites like tocopherols, flavonoids, phenylpropanoids, and ascorbate precursors were found to be enriched in the seeds of a heat-tolerant soybean cultivar [11]. In another study, changes

in the content and composition of lipids derived from the leaves of two soybean cultivars differing in heat stress tolerance after exposure to heat stress were observed [20]. Additionally, another study proposed a connection between the lipid unsaturation index, the expression levels of soybean oleate and linoleate desaturase genes, and heat stress tolerance [3]. Similarly, other biochemical pathways, such as the antioxidant defense mechanism (which increases the activity of enzymes like superoxide dismutase), phytohormone signaling (salicylic acid and abscisic acid), and carbohydrate metabolism, also function synergistically to minimize damage caused by heat stress [18,23,24]. Overall, the studies above clearly reveal that the metabolites and the pathways involved are responsible for heat stress tolerance in soybean plants.

The complex molecular mechanisms of heat stress tolerance in soybean plants are genetically regulated, with the differential expression of downstream genes linked to the aforementioned physiological and metabolic pathways playing an important role. These downstream genes include various classes of genes encoding for transcription factors, enzymes involved in biosynthetic pathways, and growth hormone metabolism (reviewed in detail by Jianing et al. [18]). Therefore, efforts have been made to characterize these genes. For instance, an earlier report identified a regulatory network of transcription factors encoding heat shock factors, ethylene-responsive element binding factors (EREB), and WRKY TFs, which showed differential expression under heat stress in soybean roots [25]. Similarly, in another study, high expression of the *DREB1* gene family (another class of TFs), dehydrins, and *LEA* genes under heat stress was also reported [26].

Fatty acid desaturases (FADs) are enzymes that introduce double bonds into lipid fatty acyl chains. Soybean has genes for both extraplastidic and plastidic FADs. The gene for extraplastidic oleate Δ12-desaturase, *FAD2*, exists as a family of eight members: *FAD2-1A, FAD2-1B, FAD2-2A, FAD2-2B, FAD2-2C, FAD2-2D, FAD2-2E*, and *FAD2-3* [27–29]. These genes are responsible for converting 18:1 extraplastidic lipids into 18:2 lipids. Similarly, the linoleate Δ-15 desaturase genes, *FAD3*, exist as a family of three members: *FAD3A*, *FAD3B*, and *FAD3C* [30,31]. These linoleate desaturase genes are responsible for converting 18:2 extraplastidic lipids into 18:3 lipids. Recently, the role of *FAD3* in modulating membrane fluidity in response to drought and salinity stress in soybean plants was demonstrated [1]. Similarly, the ectopic expression of the soybean *FAD3A* gene in rice improved germination in cold-stressed plants [32]. However, the roles of *FAD2* and *FAD3* in heat stress tolerance remain relatively unexplored, with only a few studies (including our own study; [20]) providing some evidence of these genes' involvement during heat stress, either in soybean [33] or in other crops like maize [34] and tobacco [35]. In our own earlier research, we showed a decline in the expression levels of *FAD2* and *FAD3* in soybean leaves upon exposure to heat stress [20]. In another recent study, FAD2-1, was shown to interact with GmCDPKSK5 (a type of calcium-dependent protein kinase) and assist in seed development in soybean under heat stress conditions [33]. Similarly, in maize, differential expression of *FAD* genes was observed in different tissues under heat stress [34]. In tobacco, the silencing of *FAD7* was shown to enhance the photosynthetic efficiency and membrane stability under heat stress [35].

In this study, we aimed to understand the role of the *FAD* genes in imparting heat stress tolerance in soybean plants by using mutants for these genes. Soybean plants were subjected to heat stress during flowering, and the expression of *FAD2* and *FAD3* genes was analyzed in leaves of 10 different soybean null mutants and the wild type. These mutant lines were generated by Pham et al. [36] to develop high-oleate and low-linoleate soybean genotypes. The high-oleate soybean mutants were created by pyramiding mutations in the *FAD2-1A* and *FAD2-1B* genes. However, these lines still produce linolenic acid within a range of 4–6%, which is undesirable due to the oxidative instability it imparts to the

soybean oil. To address this, mutants were developed in the *FAD3* genes, and one or more of these mutations were stacked with the high-oleic acid mutations. This approach resulted in 10 mutant lines with double, triple, and quadruple *FAD* mutations, which were used in this study to investigate the impact of heat stress on germination. Additionally, RT-PCR-based *FAD* gene expression analysis was performed on leaf tissue collected from heat-stressed and control plants to study the impact of heat stress on *FAD* gene expression.

2. Materials and Methods

2.1. Plant Material

The genetic materials used in the present study are single, double, triple, and quadruple soybean *FAD2* and *FAD3* mutants and their respective wild types (Table 1). Seeds of these mutants were obtained from the University of Missouri, Columbia, Missouri, USA, and the wild-type M92-220 from the University of Minnesota, St. Paul, Minnesota, United States of America.

Table 1. Soybean genotypes used in this study.

Name	Genotype
Williams 82	Wild type
M92-220	Wild type
S15-17812	FAD2-1A, FAD2-1B null
S17PR-345	FAD2-1A, FAD2-1B null
S17CR-172	FAD2-1A, FAD2-1B, FAD3A null
S17CR-180	FAD2-1A, FAD2-1B, FAD3A null
S17PR-662	FAD2-1A, FAD2-1B, FAD3B null
S16-17495	FAD2-1A, FAD2-1B, FAD3B null
S17PR-501	FAD2-1A, FAD2-1B, FAD3C null
S17PR-499	FAD2-1A, FAD2-1B, FAD3A, FAD3B null
S17CR-170	FAD2-1A, FAD2-1B, FAD3A, FAD3C null
S17CR-301	FAD2-1A, FAD2-1B, FAD3A, FAD3C null

2.2. Plant Growth and Heat Stress Treatment

Seeds of each genotype were planted in two-gallon pots containing Fafard®3B Mix/Metro-Mix®830 (SUNGRO Horticulture, Agawam, MA, USA). The pots were fertilized with Osmocote (18:6:12, N:P_2O_5:K_2O) at 25 g per pot and supplemented with a systemic insecticide, Marathon (a.i., Imidacloprid; OHP, Inc., Mainland, PA, USA), at 4.5 g per pot to prevent insect pest invasion. The pots were arranged in randomized complete block design with replicates. Before the heat stress treatment, all plants were grown at 30 °C during the day and 20 °C at night, with a 12 h photoperiod. At anthesis, half of the plants were exposed to 38 °C during the day and 28 °C at night for two weeks, while the remaining plants were kept at 30 °C during the day and 20 °C at night. After the heat stress, all plants were returned to the optimal growing conditions.

2.3. Gene Expression Analysis (In Silico and qRT-PCR)

In silico expression analysis was carried out using the publicly available soybean expression data for leaves, fruits, flowers, roots, and nodules (RNA-sequencing atlas of soybean genotype A81-356022 at https://legacy.soybase.org/soyseq/, accessed on 10 September 2023). After retrieval, the expression data were analyzed and plotted using MS Excel.

For qRT-PCR analysis, leaf samples were collected for RNA extraction from three biological replicates on the 7th day from the start of the heat stress treatment. Total RNA extraction was performed using MagMax Plant RNA isolation kit (Applied Biosystems, Thermo Fischer Scientific, California, USA) according to the manufacturer's instructions.

After DNase treatment, RNA was used for first-strand cDNA synthesis with the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Gene-specific primers used for the expression analysis are listed in Supplementary Table S1. Expression of *FAD* genes was normalized to *Actin 6* [20], and differential expression analysis was performed using the delta delta Ct method [37]. A heatmap showing differential expression of selected soybean *FAD* genes in leaf samples collected from plants exposed to heat stress or kept under optimal growth conditions was created using the online tool Clustvis [38].

2.4. Seed Germination Measurement

Seed germination data were recorded on six seeds from each replicate of the mutant and wild type plants, which were either exposed to heat stress during early flowering or kept under optimal growing conditions. Seeds were sown in a Fafard Sungro #3B potting mix in flat trays at 28 ± 2 °C daytime and 18 ± 2 °C nighttime temperatures under a 16 h photoperiod and germination was recorded ten days after sowing by counting the seedlings. The mean germination percentage of seeds obtained from heat-stressed plants was subtracted from the germination percentage of seeds from plants grown under optimal conditions to estimate decline in germination due to heat stress.

2.5. Statistical Analysis

The analysis of variance (ANOVA) and mean separation for seed germination was carried out using SAS v.9.4 [39]. Normalized percentage of germination decline was calculated with respect to maximum percentage of decline considered as 100%. Therefore, the percentage of decline for S17-PR170 was considered as 100% and the percentage of decline of the remaining genotypes was calculated accordingly. A correlation plot showing the relationship between gene expression (studied using qRT-PCR) and the percentage of decline in seed germination was generated using the R software package "Corrplot" [40].

3. Results and Discussion

3.1. Impact of FAD Genes on the Germination of Seeds Derived from Heat-Stressed Plants

This goal was achieved by studying the effect of mutations in the soybean *FAD* genes on the germination of seeds derived from plants exposed to heat stress during flowering, as well as those grown under optimal conditions.

An analysis of variance (ANOVA) of germination percentages revealed significant differences ($p < 0.001$) among genotypes, treatments, and the interaction between genotype and treatment (Table 2). Mean separation analysis showed that seeds obtained from plants grown under optimal conditions had a significantly higher germination percentage than those from heat-stressed plants. Seeds from all heat-exposed genotypes displayed reduced or no germination under heat stress, except for S15-17812, S16-17495, S17PR-345, S17PR-501, and S17PR-662 (Table 3). This analysis suggested that soybean plants exposed to heat stress, regardless of genotype, exhibit reduced germination; however, the severity of this effect varied among different genotypes.

While there are no reports on the impact of high-oleic-acid *FAD* mutants on seed germinability after heat exposure, Bachleda et al. [41] reported a negative effect of high-oleic-acid mutations in the soybean *FAD2-1A* and *FAD2-1B* genes on germination under cold stress. Similarly, another study demonstrated that ectopic expression of the *FAD3A* gene improved germination in cold-stressed rice plants [32]. These findings suggest that mutations in the *FAD2* and *FAD3* genes have opposing effects on germination under different stress conditions, with a negative impact on cold-stressed plants and a positive impact on the germination of seeds from heat-stressed plants, as observed in this study.

Table 2. Mean squares of germination percentage for seeds obtained from plants of 15 soybean genotypes grown under both heat stress and optimal conditions.

Source of Variation	Degree of Freedom	Germination %
Rep	5	0.09 *
Genotype (G)	11	0.41 ***
Treatment (T)	1	1.79 ***
G × T	11	0.41 ***
Error	104	0.04
R^2		0.73

*, *** indicate a significant F-test at the 0.05 and 0.001 levels of probability, respectively.

Table 3. Seeds derived from soybean *FAD* mutants exhibit a decline in germination when exposed to heat stress (HT) compared to those grown under optimal conditions (OT).

Genotype	Germination (HT)	Germination (OT)	Germination Decline	Percent Germination Decline *	Normalized Percent Germination Decline #
Williams 82 (PI518671)	0.70	0.97	0.27	27	35.06
M92-220 (WT)	0.83	0.98	0.15	15	19.48
S17CR-170	0.22	0.98	0.77	77	100.00
S15-17812	0.40	0.23	−0.18	−18	−23.38
S16-17495	0.78	0.58	−0.20	−20	−25.97
S17CR-172	0.68	0.95	0.27	27	35.06
S17CR-180	0.26	1.00	0.74	74	96.10
S17CR-301	0.28	0.95	0.67	67	87.01
S17PR-345	0.62	0.33	−0.28	−28	−36.36
S17PR-499	0.00	0.59	0.59	59	76.62
S17PR-501	0.55	0.22	−0.33	−33	−42.86
S17PR-662	0.52	0.47	−0.05	−5	−6.49

* The negative values indicate a high germination percentage under heat stress. The genotype showing the maximum percentage of decline (S17CR-170) was considered 100%. # The normalized decline for the remaining genotypes was calculated with S17-PR130 as the reference.

The differential expression of *FAD* genes under optimal growth and heat stress conditions has been previously demonstrated in soybean [20] and other crop plants, such as *Brassica* [42] and maize [34]. In our earlier work, we studied the expression of *FAD* genes in two soybean cultivars with contrasting levels of heat stress tolerance under control and heat stress conditions [20]. This study showed correspondence between the reduced accumulation of polyunsaturated lipids and the expression levels of *FAD3A* and *FAD3B* in the heat-tolerant soybean genotypes DS25-1 under heat stress conditions. Therefore, the present study validates our previous findings by further establishing a connection between *FAD* gene expression and heat stress tolerance.

Further analysis of the germination changes (Table 3) in the wild-type genotypes, Williams 82 and M92-220, revealed no significant difference in seeds derived from plants grown under optimal conditions. However, for the seeds derived from heat-stressed plants, both cultivars showed a decline in germination compared to those grown under optimal conditions. Notably, the normalized percentage decline was more pronounced in Williams 82 (35.06%) compared to M92-220 (19.58%), suggesting that M92-220 may be more tolerant to heat stress. These results are consistent with an earlier study that identified Williams 82 as heat-sensitive due to its decreased germination percentage under heat stress [43].

Interestingly, among the 10 mutants studied, five exhibited a negative germination decline percentage, indicating an increase in germination of seeds from heat-exposed plants, which may suggest heat tolerance. It would be valuable to examine these lines for other heat-tolerance-associated traits, such as yield, seed dimensions and shape, and above-

and below-ground biomass. This would help to establish a better connection among heat stress tolerance, lipidome remodeling, and *FAD* gene expression. The remaining genotypes showed a positive decline, indicating susceptibility, and this decline was more pronounced compared to the two wild types (Table 3).

Interestingly, the differences observed in the performance of the various mutant lines could be attributed to differences in their genetic backgrounds, as evidenced by the variations in *FAD* gene expression levels across the mutant lines, regardless of their mutation stacking levels (see Section 3.3). This study would have benefited if all mutant lines had been near-isogenic to each other.

3.2. In Silico Gene Expression Analysis

This analysis utilized the available expression data for *FAD2*, *FAD3*, *FAD6*, *FAD7*, and *FAD8* genes from SoyBase (https://legacy.soybase.org/soyseq/, accessed on 10 September 2023). The in silico expression data indicated significantly higher expression level for the *FAD2* and *FAD3* genes in leaves compared to other tissues (Figure 1), as well as compared to *FAD6*, *FAD7*, and *FAD8* genes. This prompted us to conduct qRT-PCR analysis for *FAD2* and *FAD3* genes using leaf tissue.

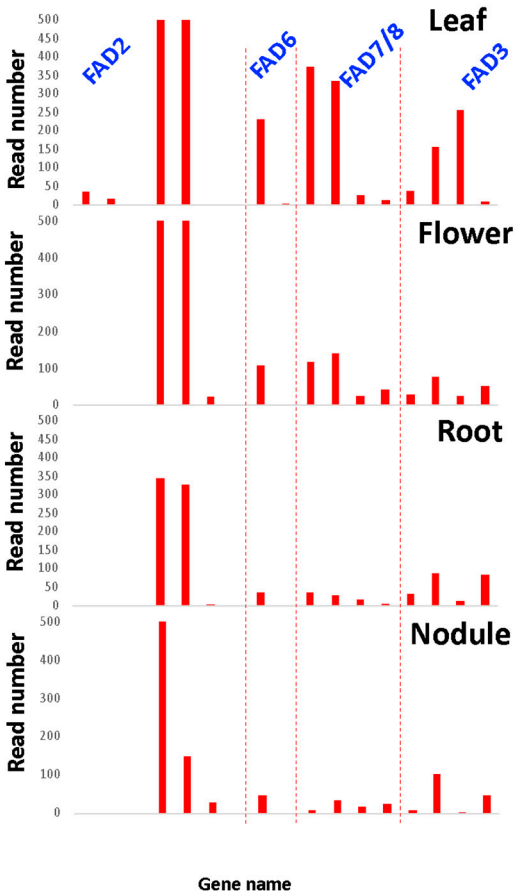

Figure 1. The expression profile of soybean *FAD* genes in the vegetative and reproductive tissues was analyzed based on in silico soybean expression data available on SoyBase (https://legacy.soybase.org/soyseq/, accessed on 10 September, 2023). The data for the in silico analysis were retrieved from SoyBase, which is based on RNA-seq experiments, as described in Ref. [44].

Additionally, our earlier study demonstrated differential expression of these genes in leaves under heat stress in both heat-sensitive and heat-tolerant soybean genotypes, and predicted association with reduced accumulation of polyunsaturated lipids under heat stress [20] Figure 1).

3.3. qRT-PCR Analysis of FAD2 and FAD3 Expression Level Changes Under Heat Stress

In most cases, the results showed either downregulation (<−2 fold) or no significant change in expression under heat stress compared to the control (Figures 2 and S1). *FAD-3A* showed upregulation in 8 of the 12 genotypes (10 mutants and 2 wild types), but not S15-17812, S17PR-662, S16-17495, and S17PR-501. *FAD-3B* showed downregulation in 9 of the 12 studied genotypes, but not M92-220, S17CR-172, and S17CR-170. Similarly, *FAD-3C* showed downregulation in 9 out of the 12 genotypes, except S17CR-172, S17PR-662, and S17CR-170. On the other hand, *FAD-2-1A* showed downregulation in all genotypes except for S15-17812. Similarly, *FAD-2-1B* showed downregulation in all genotypes except for S17CR-172 and S17PR-501. Meanwhile, somewhat mixed results were observed for *FAD-2-2C*, which showed upregulation in S16-17495, and *FAD-2-3*, which showed upregulation in seven mutants and downregulation in five mutant lines (Figures 2 and S1).

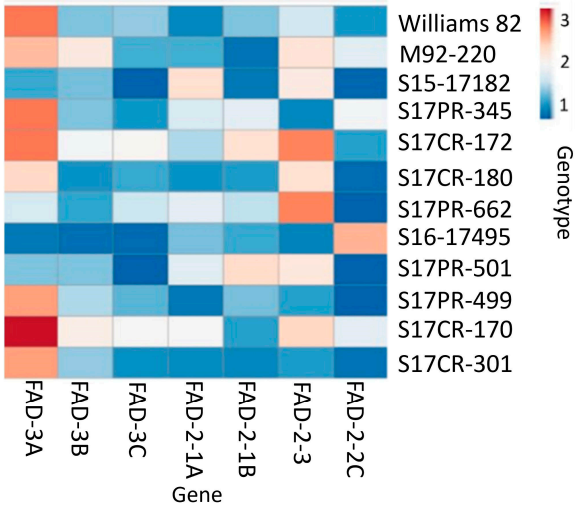

Figure 2. A heatmap showing the differential expression of selected soybean *FAD* genes in leaf samples collected from the wild-type (Williams 82) and its *FAD* mutants under heat stress conditions (38 °C during the day and 28 °C at night with a 12 h photoperiod), relative to optimal growth conditions (30 °C during the day and 20 °C at night, with a 12 h photoperiod), as determined by qRT-PCR.

The reduced expression of *FAD3* genes in the leaves of heat-stressed plants directly or indirectly impacts photosynthesis by affecting membrane stability and fluidity, which in turn influences assimilate accumulation in cotyledons. This is expected to affect the accumulation of reserves, as well as the enzymes and energy resources needed during germination.

While the downregulation of genes is expected due to heat stress, there are instances where the upregulation of these genes has been observed. Of the seven genes studied, two—*FAD3A* and *FAD2-3*—showed higher expression (>2-fold) in 6 out of 12 lines. The expression of *FAD3A* was particularly pronounced (7-fold higher) in the mutant S17CR-170 under heat stress, despite the fact that it carries a point mutation in this gene. Similarly, other mutants such as S17-CR172, S17-PR499, and S17CR-170 also exhibited high expression levels for either the soybean *FAD-3A* or *FAD-2-3* genes, or both. All these genotypes have substitutions in the target genes, not deletions or gene truncations [36].

Additionally, *FAD* genes belong to a large gene family consisting of 75 members, as revealed by a genome-wide analysis of these genes in three soybean genomes [45]. Therefore, compensatory expression leading to a cumulative increase in gene expression is a plausible explanation for the observed spike. Another possible explanation is that substitutions rendering enzymes dysfunctional or with reduced functionality often result in overproduction of the transcript to compensate for the loss of function.

Furthermore, the germination analysis of seeds derived from heat-stressed plants showed a significant decline in germination ($p < 0.01$), which was positively correlated (up to 0.48) with changes in the expression pattern of four genes (*FAD3-A*, *FAD3-B*, *FAD3-C*, and *FAD2-3*) (Figure 3). This suggests that the *FAD* genes act as negative regulators of seed germination in the heat-exposed plants, where the high expression of these genes contributes to reduced germination through an unknown mechanism. An interesting observation is the high expression of the *FAD2-2C* gene in the mutant line S16-17495, correlates with increased germination under heat stress. This may indicate a mechanism where the overproduction of 18:2 fatty acids facilitates the cycling of polyunsaturated fatty acids during membrane remodeling under heat stress. However, the exact mechanism behind the high germination of selected *FAD* mutants under heat stress remains unclear. Similar high performance (e.g., high yield) under heat stress has been observed in other oilseed crops, such as peanuts [46]. As previously mentioned, heat stress is a complex trait controlled by a network of genes, including transcription factors, heat shock proteins, regulatory proteins, and non-coding RNAs [47]. Future detailed studies involving the aforementioned gene will provide better insights into the molecular mechanism of heat tolerance in soybean and clarify whether selected *FAD* genes are actually regulated by other transcription factors or non-coding RNAs. Additionally, other mutants, such as S15-17182, S17-PR345, S17PR-501, and S17PR-662, also showed high germination rates after exposure to heat stress, with one to three *FAD* genes (*FAD3-A* in S17-PR345; *FAD2-3* in S17PR-662; *FAD2-3* and *FAD2-1A* in S15-17182; and *FAD2-1B* and *FAD2-3* in S17PR-501; and *FAD-3A*, *FAD-3B*, and *FAD2-3* in S17CR-170) exhibiting high expression in these lines under heat stress.

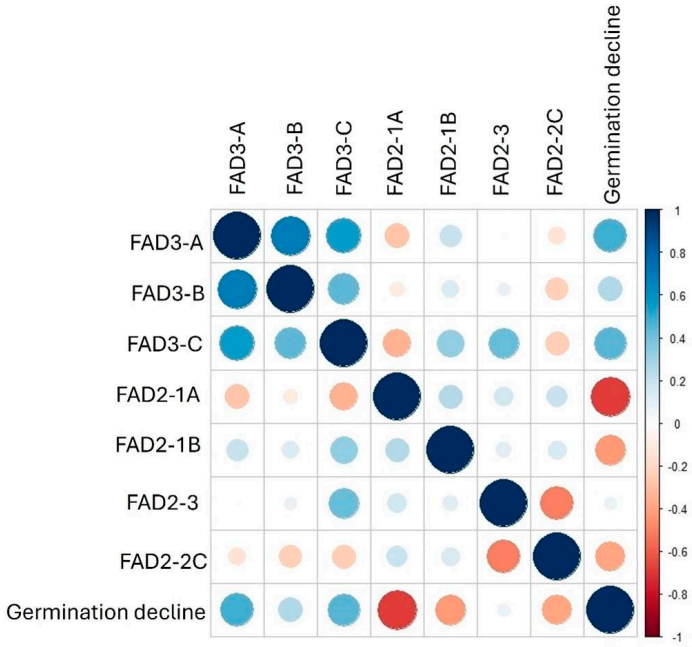

Figure 3. A correlation plot showing the relationship between gene expression, as studied using qRT-PCR, and germination decline. The size and color of the filled circles is proportional to the level of the Pearson correlation coefficient; the scale on the right indicates the level of correlation. A higher intensity of blue color indicates a highly positive correlation coefficient, while a higher intensity of red indicates a low correlation coefficient.

4. Conclusions

The present study aimed to understand the role of *FAD* genes in heat stress tolerance in soybean plants, focusing on the germinability of seeds from heat-exposed plants. This investigation used 10 different *FAD* mutant lines (Table 1) alongside their respective wild

types. These mutant lines, which include double, triple, and quadruple *FAD* mutations, provide valuable material for assessing the impact of *FAD* genes on heat stress tolerance in soybean plants. Initial screening of these lines was conducted to evaluate the variability in germination percentage of seeds derived from plants grown under optimal conditions and those exposed to heat stress during flowering. To further support these findings, RT-PCR-based gene expression analysis of the *FAD* genes was performed on leaf tissue collected from heat-stressed and control plants, allowing examination of the impact of heat stress on *FAD* gene expression. The results revealed a negative correlation between the expression patterns of specific *FAD3* (*FAD3-A*, *FAD3-B*, and *FAD3-C*) and *FAD2* (*FAD2-3*) genes under heat stress and the seed germinability of heat-exposed plants. These observations suggest that *FAD3* genes may influence lipid unsaturation level, membrane stability, and, consequently, the stability of the photosynthetic machinery, the accumulation of reserves, and germination.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/crops5010002/s1, Figure S1: Bar diagrams showing the differential gene expression fold changes for (a) *FAD2-1A*; (b) *FAD2-2*; (c) *FAD2-3*; (d) *FAD2-1B*; (e) *FAD3A*; (f) *FAD3B*; and (g) *FAD3C*. These diagrams represent 12 different soybean genotypes under optimum and heat stress conditions. The error bars indicate the standard error of the mean (SEM) in the fold changes. The blue bars in each figure represent the expression delta Ct values under optimum conditions, while the orange bars indicate the expression delta Ct values under heat stress conditions; Table S1: List of gene-specific primers for qRT-PCR analysis.

Author Contributions: J.O.T. performed the experiment and collected the data jointly with H.P.I., Z.T.J., S.N. (Salman Naveed) and E.N.; G.S. and J.O.T. analyzed the data; J.O.T. wrote the first draft of the manuscript jointly with G.S.; S.R. conceived the experiment and edited and finalized the manuscript jointly with S.N. (Sruthi Narayanan). All authors have read and agreed to the published version of the manuscript.

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