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Impact of Mutations in Soybean Oleate and Linoleate Desaturase Genes on the Germinability of Seed from heat-Stressed Plants at the Anthesis Stage

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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 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, exposure to heat stress during anthesis is known to significantly reduce seed germination. In silico expression analysis indicated high levels of expression of the soybean *FAD2* and *FAD3* genes in 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 mutant S17CR-170. Correlation analysis revealed a positive correlation (0.5) 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

1. Introduction

Soybean (*Glycine max* L., 2*n* = 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 raw material for various medical and industrial purposes (Singh et al. 2022). Soybean is the leading oilseed crop in the U.S., accounting for about 90% of the total oilseed production (Economic Research Service U.S. Department of Agriculture 2021). 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 the decline attributed partly to global warming-induced decline in productivity and primarily to increased soybean acreage in Brazil (Rustgi et al. 2021; https://www.statista.com/statistics/263926/soybean-production-in-selected-countries-since-1980/). 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

 doi:10.20944/preprints202409.1860.v1

maturing soybean varieties are planted in zones typically suitable for late maturity varieties with an aim to skip heat stress during anthesis by flowering early. This strategy, however effective, presented a unique challenge as plants sown under ESPS undergo seed maturation under heat stress (Bellaloui et al. 2009).

Heat stress can occur at any developmental stage during soybean growth; however, it is most damaging during anthesis and seed development (Poudel et al. 2023). For instance, according to Lobel and Asner (2003), an increase in temperature by 1°C, result in up to a 17% decline in soybean yield. Similarly, according to Schauberger et al. (2017), soybean yield under rainfed conditions decline 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 reduction in pollen viability, seed weight, and seed size. It also results in 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 (Heatherly and Spurlock 1999; Smith et al. 2008; Chebrolu et al. 2016; Hamayun et al. 2021).

Heat stress in crop plants generally results in several complex biochemical and physiological alterations that limit productivity (Kosina et al. 2007). Specifically, heat stress reduces assimilate production and translocation, increases carbohydrate starvation, and causes high respiration, especially under high nighttime temperatures (Snider et al. 2010). 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 leaf, pod, sepal, anther, stigma (Sinha et al. 2023), seedlings (Wang et al. 2018) and at various developmental stages, such as leaves at the reproductive stage (Xu et al. 2020). 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. Lipidomic, proteomic, and metabolomic studies were carried out to further understand the molecular mechanisms of heat tolerance (Narayanan et al. 2020; Krishnan et al. 2020; Chebrolu et al. 2016). For instance, a detailed study of leaf metabolites revealed the importance of sugar and nitrogen metabolism under heat and drought stress in soybean (Das et al. 2017). Similarly, temperature- and genotype-specific differences were observed in 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 heat-tolerant soybean cultivar (Chebrolu et al. 2016). 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 (Narayanan et al. 2020). Additionally, the study proposed a connection between the lipid unsaturation index, the expression levels of soybean oleate and linoleate desaturase, and heat stress tolerance (Rustgi et al. 2021).

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* (Schlueter et al. 2007; Román et al. 2012; Dar et al. 2017). These genes are responsible for converting 18:1 extraplastidic lipids to 18:2 lipids. Similarly, the linoleate Δ-15 desaturase genes, *FAD3*, exist as a family of three members: *FAD3A*, *FAD3B*, and *FAD3C* (Andreu et al. 2010; Chi et al. 2011). These genes (linoleate desaturase) are responsible for converting 18:2 extraplastidic lipids to 18:3 lipids. Recently, the role of *FAD3* in modulating membrane fluidity in response to drought and salinity stress in soybean was demonstrated (Singh et al. 2022). Similarly, the ectopic expression of the soybean *FAD3A* gene in rice improved germination in cold-stressed plants (Wang et al. 2019). However, the roles of *FAD2* and *FAD3* in heat stress tolerance remain relatively unexplored.

In this study, we aimed to understand the role of the *FAD* genes in imparting heat stress tolerance in soybean 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

 doi:10.20944/preprints202409.1860.v1

soybean null mutants and the wild type. These mutant lines were generated by Pham et al. (2012) 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 in 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 wildtype 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 2-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 pests 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-hour 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)

 doi:10.20944/preprints202409.1860.v1

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 A81-356,022 at http://www.soybase.org/soyseq). After retravel, the expression data was analyzed and plotted using MS Excel.

For qRT-PCR analysis, leaf samples were collected for RNA extraction from three biological replicated on the 7th day from the start of the heat stress treatment. Total RNA extraction was performed using TRIzol reagent (Invitrogen by ThermoFischer Scientific Ltd., 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, USA). Gene-specific primers used for the expression analysis are listed in supplementary Table S1. Expression of *FAD* genes was normalized to *Actin*, and differential expression analysis was performed using the delta delta Ct method (Livak et al. 2001). A heatmap showing differential expression of selected soybean *FAD* genes in leaf samples collected from plants exposed to heat stress or kept at optimal growth conditions was created using online tool Clustvis (Metsalu et al. 2015).

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 obtained from heat-stressed and non-stressed plants were sown in pots in the greenhouse in the randomized complete block design in six replicates. Seeds were sown on Fafard Sungro #3B potting mix in flat trays at 28 ± 2°C daytime and 18 ± 2°C night-time temperatures under 16h 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.

Statistical Analysis

The analysis of variance (ANOVA) and mean separation for seed germination was carried out using SAS v.9.4 (SAS Institute 2016). Normalized percent germination decline was calculated with respect to maximum percent decline considered as 100%. Therefore, the percent decline for S17-PR170 was considered as 100% and the percent decline of remaining genotypes was calculated accordingly. A correlation plot showing the relationship between gene expression (studied using qRT-PCR) and the percentage decline in seed germination was generated using the R software package "Corrplot" (Wei and Simko 2024).

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 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 varies among different genotypes.

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

 doi:10.20944/preprints202409.1860.v1

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

*, *** indicate significant F-test at 0.05 and 0.001 level of probability, respectively.

Table 3. Germination decline in seed derived from soybean *FAD* mutants exposed to heat stress (HT) compared to those grown under optimal (OT) conditions.

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 high germination percentage under heat stress. #The genotype showing the maximum percent decline (S17CR-170) was considered as 100% and the normalized decline for the remaining genotypes was calculated by considering the S17-PR130 as a reference.

While there are no reports on the impact of high oleic acid *FAD* mutants on seed germinability after heat exposure, Bachleda et al. (2017) 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 (Wang et al. 2019). 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.

The differential expression of *FAD* genes under ambient and heat stress conditions has been previously demonstrated in soybean (Narayanan et al. 2020) and other crop plants, such as Brassica (Shaheen et al. 2023) and maize (Zhao et al. 2019). 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 (Narayanan et al. 2019). This study showed correspondence between the reduced accumulation of polyunsaturated lipids and the expression levels of *FAD3A* and *FAD3B* in

 doi:10.20944/preprints202409.1860.v1

the heat-tolerant soybean genotypes DS25-1 under heat stress conditions. Therefore, the present study served as a validation of 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 plant grown under optimal conditions. However, for the seeds derived from heat-stressed plants, both cultivars showed a decline in germination compared to the control. 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 (Khan et al. 2007).

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 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 next section). 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://www.soybase.org/soyseq/). 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.

 doi:10.20944/preprints202409.1860.v1

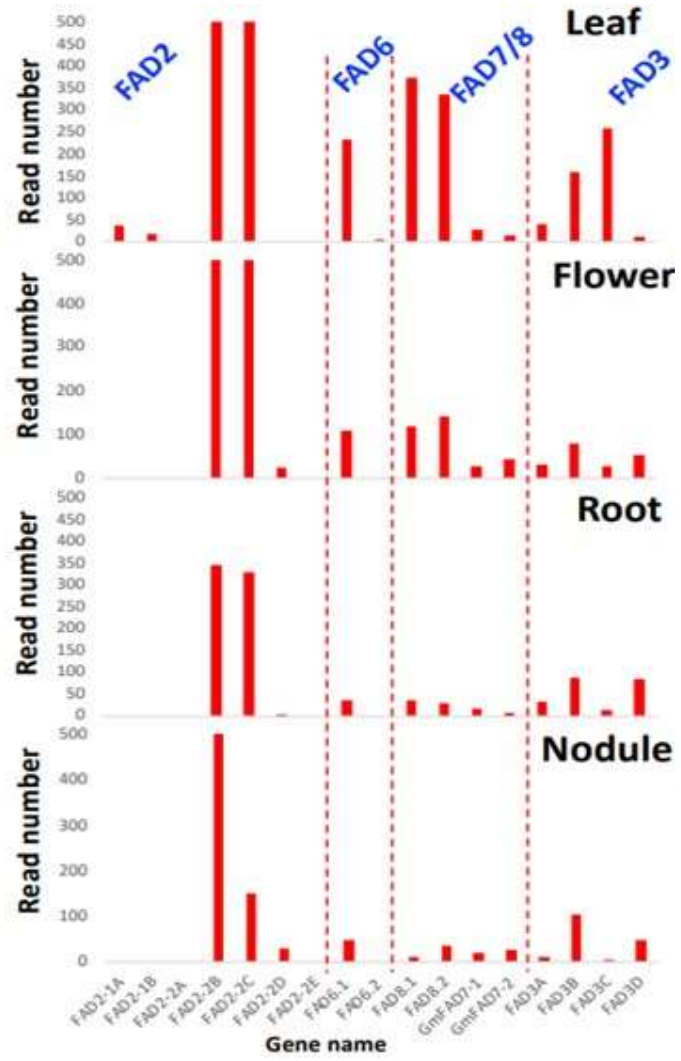

Figure 1. Expression profile of the soybean *FAD* genes in vegetative and reproductive tissues, based on in silico soybean expression data available in Genevestigator® software (Hrus et al. 2008).

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 (Narayanan et al. 2020; 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 control (Figure 2). *FAD-3A* showed upregulation in eight of the 12 genotypes (10 mutants and two wild types), except S15-17812, S17PR-662, S16-17495, and S17PR-501. *FAD-3B* showed downregulation in nine of the 12 studied genotypes, except 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 S15-17812. Similarly, *FAD-2-1B* showed downregulation in all genotypes, except S17CR-172 and S17PR-501. Whereas somewhat mixed results were observed with *FAD-2-2C*, showed upregulation in S16-17495, and *FAD-2-3*, showed upregulation in seven mutants and downregulated in five (Figure 2).

 doi:10.20944/preprints202409.1860.v1

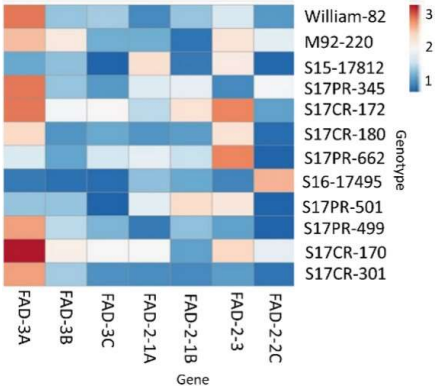

Figure 2. Heatmap showing the differential expression of selected soybean *FAD* genes in leaf samples collected from plants exposed to heat stress, relative to optimal growth conditions, as determined by qRT-PCR.

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 downregulation of genes is expected due to heat stress, there are instances where upregulation of these genes has been observed. Of the seven genes studied, two—*FAD3A* and *FAD2-3*—showed higher expression (>2-fold) in six out of 12 lines. The expression of *FAD3A* was particularly pronounced (7-fold higher) in the mutant S17CR-170 under heat stress, despite 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 (Pham et al. 2012).

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 (Zhang et al. 2021). 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.

Further, the germination analysis of seeds derived from heat stressed plants showed a significant decline in germination ($P<0.01$), which was positively correlated (0.5^{**}) 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, which correlates with high germination under heat stress. This may indicate a mechanism where the overproduction of 18:2 fatty acids facilitate 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 remain unclear. Similar high performance (e.g., high yield) under heat stress has been observed in for other oilseed crops, such as peanuts (Akbar et al. 2017). As previously mentioned, heat stress is a complex trait controlled by a network of genes, including transcription factors, heat shock proteins, regulatory proteins, non-coding RNAs (Huang et al. 2022). 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 1 to 3 *FAD* genes (*FAD3-A* in S17-PR345; *FAD2-3* in S17PR-

 doi:10.20944/preprints202409.1860.v1

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.

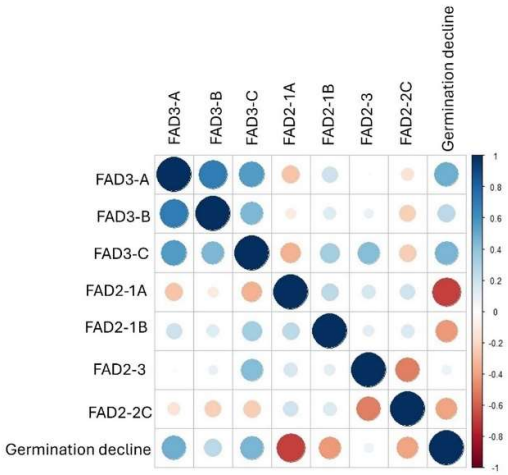

Figure 3. A correlation plot showing the relationship between gene expression, as studied using qRT-PCR, and phenotypic traits such as seed weight and the percent decline in seed germination.

Conclusion

The present study aimed to understand the role of *FAD* genes in heat stress tolerance in soybean, particularly in the germinability of seeds from heat-exposed plants, using 10 different *FAD* mutant lines (Pham et al. 2012; Table 1) along with their respective wild types. These mutant lines, which have double, triple, and quadruple *FAD* mutations, serve as important material for investigating the impact of *FAD* genes on heat stress tolerance in soybean. Initial screening of these lines was conducted to assess the variability in germination percentage of seeds derived from plants grown under optimal conditions and exposed to heat stress during flowering. The results were further supported by RT-PCR-based gene expression analysis of the *FAD* genes, performed on leaf tissue collected from heat-stressed and control plants to examine 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 seed germinability of heat-exposed plants. These observations link the impact of *FAD3* genes to the unsaturation level of lipids, membrane stability, and, consequently, the stability of the photosynthetic machinery, accumulation of reserves, and germination.

Author Contributions: JOT performed the experiment and collected the data jointly with HPI, ZTJ, SN and EO. GS analyzed the data and wrote the first draft of manuscript jointly with JOT; SR conceived the experiment, edited and finalized the manuscript jointly with SN.

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Data Availability Statement: All the data related to manuscript is presented in the main text.

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Conflicts of Interest: Authors declare no conflict of interest, financial or otherwise.

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