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Environmental Stability of Elevated α-Linolenic Acid Derived from a Wild Soybean in Three Asian Countries

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Abstract: Soybean has been consumed in Asia traditionally as a staple food. Soybean can be a source of essential fatty acids—linoleic (18:2, ω-6) and α-linolenic acid (18:3, ω-3)—for humans. Intake of fatty acids with high ω-3 concentration or low ω-6/ω-3 ratios is more desirable for human health. However, in soybean, the unsaturated fatty acids are less stable than the saturated fatty acids in different environments. The objective of the present study is to expand the understanding of the environmental stability of elevated α-linolenic acid of soybean genotypes with alleles from wild soybean grown in three Asian countries. The results highlighted an environmental effect on the accumulation of 18:3, following the growth of soybean genotypes with elevated α-linolenic acid in eight environments. Particularly, temperature influenced the accumulation of 18:3 concentration. The soybean genotype, UT-385-4-4, produced the highest 18:3 concentration and is more stable than all the other soybean genotypes, excluding PT-100-3. UT-385-4-4 is a potential genetic resource to develop novel cultivars with high 18:3 concentration, which could be dietary sources of plant-derived ω-3 fatty acids.

Keywords: wild soybean; α-Linolenic acid; Omega-3; subtropical region

1. Introduction

Soybean (*Glycine max* (L.) Merr.) seeds contain 40% protein and 20% oil. Because of its seed composition, it is one of the most economically important oil crops globally. Sixty-one percent of world oil seed production was from soybean seed, followed by rapeseed, sunflower, and peanut [1]. Generally, soybean oils are composed of triacylglycerols, with a glycerol backbone attached to three fatty acids and fatty acid compositions that influence soybean oil uses [2]. Five major fatty acids are found in soybean oil: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and α-linolenic acid (18:3). The fatty acids in cultivated soybean oil are 11% of 16:0, 4% of 18:0, 23% of

18:1, 55% of 18:2, and 8% of 18:3 [3]. Depending on the existence of double bond(s) in the carbon chains of fatty acids, oils either have saturated or unsaturated fatty acids. The saturated fatty acids are 16:0 and 18:0, whereas the unsaturated fatty acids in soybean oil are divided into two categories: mono-unsaturated fatty acid (18:1) and polyunsaturated fatty acid (18:2 and 18:3).

In 2018, 56.3 million metric tons of soybean oil were consumed globally [1]. However, trans-fats are generated during the soybean oil hydrogenation process, which improve its oxidative and heat stability. Numerous studies on trans-fat have concluded that it poses risks to human health and could cause coronary heart disease and increase cholesterol levels [4–6]. Following an increase in awareness about the potential adverse effects of trans-fats, the Food and Drug Administration (FDA, Silver Spring, MD, USA) introduced a food labeling regulation requiring the indication of the trans-fat contents on food nutrition fact labels [7]. The generation of trans-fats in soybean oil has discouraged the use of soybean oil in foods. To address the generation of trans-fats in the course of hydrogenation and to improve oxidative stability, soybean breeders have been challenged to reduce the 18:3 concentration from 8% to 1% in soybean oil [8–12].

Although in Western countries soybean production focuses on providing high protein meals for livestock and the manufacture of vegetable oils, in many Asian countries, traditionally, soybean has been used as a staple food that is consumed as soy milk, tofu, soy sprout, fermented soy foods, and soy sauce [13,14]. Nevertheless, the consumption of soy foods has been increasing in North America, following the recognition of the health benefits of soy foods.

When soybean is consumed directly as food, polyunsaturated fatty acids such as linoleic (18:2, ω-6) and α-linolenic acid (18:3, ω-3) are essential fatty acids and are the precursors of eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). Such fatty acids prevent inflammation, cardiovascular diseases, and Alzheimer's disease, as well as promote fetal development [15,16]. In addition, numerous studies have concluded that a higher intake of ω-3 fatty acid or fatty acids with relatively low ω-6/ω-3 ratios is appropriate from a human health perspective [17–19].

The ω-6/ω-3 ratios of cultivated soybean generally range from 6:1 to 7:1 [20,21]. The mean 18:3 concentration in 18 cultivated soybean accessions with elevated α-linolenic acid (EALA) from the USDA Soybean Germplasm Collection grown in South Korea ranged from 6.5 to 10.7% in soybean seed oil. However, most wild soybean (*Glycine soja Sieb.* and Zucc.) seeds have oils with almost two-fold (~15%) the 18:3 concentration of cultivated soybean oil, and therefore the ω-6/ω-3 ratios are approximately 4:1 in wild soybean oil [20,22–24]. In addition, Pantalone et al. [25] suggested that the accumulation of a high concentration of 18:3 in wild soybean was due to a different set of desaturate alleles controlling 18:3 in cultivated soybean. Therefore, it is necessary to develop novel cultivated soybean lines with high 18:3 concentration using wild soybean as a genetic resource for increasing 18:3 concentration and lowering ω-6/ω-3 ratios [20,26].

As the instability of fatty acids is a source of concern, many studies have explored genotype–environment interactions regarding fatty acids in soybean seeds. Studies have reported that temperature influences fatty acid profiles, particularly in the case of unsaturated fatty acids [27–32]. Oliva et al. [33] reported that soybean genotypes with higher 18:3 concentration were less stable across 10 growth environments. However, three recombinant inbred lines (RILs) with EALA concentration from an interspecific cross between *G. max* and *G. soja* had stable 18:3 concentration across different environments [20]. Therefore, it is important to understand the stability of 18:3 concentration using EALA lines in diverse geographical locations. Stable EALA lines can be used as food containing plant-derived ω-3, which have high protein concentrations, for people living in countries with risks of inadequate ω-3 intake. The objective of the present study was to enhance our understanding of the environmental stability of EALA concentration in soybean lines containing alleles from a wild soybean, PI 483463, grown in three Asian countries, including two subtropical nations.

2. Materials and Methods

2.1. Soybean Genotype

To analyze fatty acid profiles across different environments, 15 soybean accessions were tested in the present study, including 12 soybean lines with EALA and three check cultivars with normal 18:3 concentration (Table 1). Soybean cultivars Williams 82 [34], Pungsannamul [35], and Uram [36] were used as check cultivars for the study. Previous studies developed a RIL166 with a high 18:3 (~14%) concentration from a cross of PI483463 (wild soybean with 15.4% 18:3) [20,23] and Hutcheson (PI 518664, cultivated soybean with 9.2% 18:3) [37]. In addition, a progeny plant, TR166-552, was developed from a cross between Taekwang (cultivated soybean with 8% 18:3) [35] and RIL166. TR166-552, with ~15% 18:3, was selected from a segregating population based on fatty acid profiles using gas chromatography in 2011. In the present study, soybean genotypes were obtained from two independent cross-combinations (Pungsannamul × TR166-522 and Uram × TR166-522) at the Experiment and Practice Fields of Kyungpook National University (KNU), Gunwi, Republic of Korea, in the summer of 2012. F_1 plants were planted in 2013, and F_2 plants were planted in 2014. Two generations from F_3 to F_4 were advanced at the winter nursery of Vietnam using the Single Seed Descent method. The $F_{4:5}$ lines were planted at KNU to produce $F_{4:6}$ in 2016. The fatty acid profiles of the harvested $F_{4:7}$ seeds were assessed using gas chromatography in 2017. Based on the fatty acid profiles in the soybean oil, lines with high 18:3 concentration were selected. Five lines from a cross between Pungsanamul and TR166-522, and seven lines from a cross between Uram and TR166-522, were used in further stability analyses.

Table 1. Nomenclature, pedigree, generation, and trait information for soybean accession used in this study.

Name	Pedigree Information [a)]	Generation	Trait
PT-65-4	Pungsannamul x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
PT-98-1-4	Pungsannamul x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
PT-100-3	Pungsannamul x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
PT-1190-2	Pungsannamul x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
PT-1133-4-1	Pungsannamul x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
UT-46-3-3	Uram x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
UT-124-3-4	Uram x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
UT-223-1-1	Uram x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
UT-223-3-2	Uram x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
UT-385-4-4	Uram x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
UT-475-4-4	Uram x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
UT-480-3-2	Uram x TR166-552	$F_{4:8}$ / $F_{4:9}$	Elevated 18:3
Pungsannamul	Check		Normal 18:3
Uram	Check		Normal 18:3
Williams82	Check		Normal 18:3

[a)] Pungsannamul, Uram, and Williams 82 are cultivars with a normal level of α-linolenic acid (18:3). TR166-552 is a progeny plant having elevated concentration of 18:3 from RIL166 and Taekwang. RIL166 is a line from a cross of Hutcheson and PI 483463. Taekwang is a Korean cultivar and Hutcheson is a US cultivar. Both Taekwang and Hutcheson have a normal level of 18:3. PI 483463 is a wild soybean with elevated concentration of 18:3 (Asekova et al., 2014; Ha et al., 2014).

2.2. Growth Condition

To understand the stability of the 18:3 concentration, the entire set of 15 accessions were grown in four locations, including Dong Thap, Dan Phuong District, Hanoi, Vietnam (21°7′ N), Maize and Cash Crops Research Center's experimental field in Laos (18°8′ N), Experiment and Practice Fields of Kyungpook National University, Gunwi, Republic of Korea (36°11′ N), and affiliated experiment and practice fields of Chonnam National University, Gwangju, Republic of Korea (35°17′ N) (Table 2). Soybean genotypes that were grown in the eight environments with specific locations and planting

dates are listed in Table 2. The experimental design was a randomized complete block design with two replicates. The soybeans were planted in hills in rows 70 cm apart with 50 cm spacing between hills. There were some missing data in the present analysis due to poor germination. Plant maturity (R8) was assessed in each plot in Vietnam and Laos, and seeds were harvested in bulk from each plot [38]. Approximately five seeds from a plot of all soybean lines were used for phenotypic fatty acid profile analyses [20–23,39].

Table 2. Planting locations, years, latitude, and planting dates at each of four locations over two years.

Environments.	Location	Year	Latitude	Planting Date
E1	Hanoi, Vietnam	2018	21°7′ N	20 Sep. 2018
E2	Hanoi, Vietnam	2019	21°7′ N	7 Feb. 2019
E3	Vientiane, Laos	2018	18°8′ N	2 Sep. 2018
E4	Vientiane, Laos	2019	18°8′ N	10 Aug. 2019
E5	Gunwi, Republic of Korea	2018	36°11′ N	22 May 2018
E6	Gunwi, Republic of Korea	2018	36°11′ N	19 Jun. 2018
E7	Gwangju, Republic of Korea	2018	35°17′ N	29 May 2018
E8	Gwangju, Republic of Korea	2018	35°17′ N	29 Jun. 2018

2.3. Phenotype Determination by Gas Chromatography

Fatty acid profiles (16:0, 18:0, 18:1, 18:2, and 18:3) in soybean seeds were expressed as a percentage of the total fatty acids of seed based on gas chromatography data obtained using an Agilent series 7890A capillary gas chromatograph equipped with a flame ionization detector at 250°C (Agilent Technologies Inc., Wilmington, DE, USA) for fatty acid methyl esters in extracted oil. The oil was extracted by placing crushed seeds in a 1.5 mL solution of chloroform, hexane, and methanol (8:5:2, v/v/v) for ~12 hours. A 100 µL derivatization solvent was mixed with 75 µl of a methylation reagent (0.25 M methanolic sodium methoxide:petroleum ether:ethyl ether [1:5:2, v/v/v]). The samples were diluted with hexane to approximately 1 mL. Five fatty acids were separated on a DB-FFAP capillary column (30 m × 0.25 mm, 0.25 µm, Agilent Technologies Inc., Wilmington, DE, USA). Standard fatty acid mixtures (Fame #16, RESTEK) were used as calibration reference standards.

2.4. Data Analysis

All statistical analyses in this study were conducted in SAS v9.4 (SAS Institute, 2013). Analysis of variance (ANOVA) was conducted to evaluate differences among eight environments using PROC GLM in SAS. Mean differences among the soybean genotypes were analyzed using Fisher's Least Significant Difference (LSD) test at $p = 0.05$. To compare the stability of genotypes for 18:3 concentration among different environments, the ranges of 18:3 concentration, coefficients of variation (CV), and stability coefficients (b_E) were used as stability parameters [40]. The stability coefficient was calculated from the regression of the mean of the concentration in a soybean genotype in an environment based on an environmental index. The environmental index is the mean of each concentration of all genotypes at a specific environment minus the mean 18:3 concentration of all genotypes averaged across the eight environments. Genotypes with stability regression coefficients closer to zero were more stable, whereas those that deviated significantly from zero (either positive or negative) were considered less adaptable to change across environments. PROC REG in SAS was used to calculate the stability regression coefficients (b_E). A genotype plus genotype by environment (GGE) plot was constructed by an R package named GGE in R Studio [41]. The polygon view of GGE plot analysis was used to visually assess which lines were the best for the accumulation of 18:3 concentration in specific environments. This plot showed the first principal component (PC1) against the second principal component (PC2).

3. Results

The present study investigated the stability of 18:3 concentration in soybean seeds, which were derived from wild soybean, across eight environments. We determined the amounts of fatty acids extracted from mature seeds of the soybean genotypes grown in different field environments. ANOVA was conducted to determine the influence of environmental and genetic effects on each fatty acid (Table 3). Genotype, environment, and genotype × environment interactions influenced each evaluated fatty acid in the soybean lines in the eight environments significantly ($p < 0.001$). A larger mean square value indicates a greater influence on fatty acid accumulation (Figure S1). The primary factor in the 16:0 and 18:0 was the environmental factor, with 66.55% and 69.3% of the total mean squares, respectively. Although the values of the genotypic effect for the 18:1 and 18:2 were 44.1% and 42.4%, respectively, the environment effect was the most significant factor influencing the accumulation of 18:1 and 18:2. The 73.7% of total variation for the accumulation of the 18:3 was due to an environmental factor. Overall, an environmental effect was the key factor influencing fatty acid accumulation.

Table 3. Mean squares from analysis of variance (ANOVA) of each measured fatty acid profiles for soybean genotypes in four locations over 2 years.

Source.	df	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid	α-linolenic Acid
Genotype (G)	14	3.15 ***	0.45 ***	223.84 ***	103.83 ***	29.11 ***
Environment (E)	7	8.27 ***	1.46 ***	234.98 ***	108.40 ***	90.96 ***
Replication in E	8	0.60 ***	0.06	4.94	4.26	0.61
G*E	95	0.43 ***	0.14 ***	43.55 ***	28.38 ***	2.66 ***
Error	98	0.12	0.05	4.56	3.01	0.63

*** Significant at the 0.001 probability level.

Mean fatty acid concentrations in EALA soybean genotypes and check cultivars across the eight environments are listed in Table 4. The mean 16:0 and 18:0 concentrations in soybean lines ranged from 10.5% to 12.0% and 3.0% to 3.6%, respectively. Soybean genotypes contained 16.1%–30.9% of 18:1, 47.2%–57.6% of 18:2, and 7.9%–12.9% of 18:3. On average, EALA lines contained 11.5% 18:3, which is 3.2% higher than the concentration in the check cultivated soybeans (8.3%) across the eight environments. Therefore, the ω-6/ω-3 ratio in the EALA lines ranged from 4.4 to 5.6, which were lower than those of the check cultivars (6.0 to 6.3).

Table 4. Concentration of fatty acids of soybean genotypes averaged in eight different environments.

Genotype	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid (ω-6)	α-linolenic Acid(ω-3)	Ratio of ω-6 and ω-3
	Mean (%) ± Standard Deviation					
PT-65-4	11.7 ± 0.5	3.3 ± 0.3	17.4 ± 3.0	57.3 ± 2.7	10.3 ± 1.7	5.6
PT-98-1-4	11.2 ± 0.3	3.2 ± 0.5	17.0 ± 2.4	57.0 ± 2.1	11.6 ± 1.6	4.9
PT-100-3	11.6 ± 0.7	3.0 ± 0.4	17.4 ± 3.1	57.6 ± 2.6	10.5 ± 1.2	5.5
PT-1190-2	12.0 ± 0.4	3.1 ± 0.3	17.9 ± 7.3	55.8 ± 5.1	11.3 ± 2.7	4.9
PT-1133-4-1	12.0 ± 0.8	3.2 ± 0.4	17.0 ± 1.9	57.1 ± 1.5	10.7 ± 1.6	5.3
UT-46-3-3	11.6 ± 0.8	3.6 ± 0.4	18.3 ± 4.1	55.3 ± 2.7	11.2 ± 2.1	4.9
UT-124-3-4	11.5 ± 0.3	3.4 ± 0.5	16.7 ± 4.0	56.8 ± 2.6	11.5 ± 2.4	4.9
UT-223-1-1	11.3 ± 1.1	3.3 ± 0.4	19.8 ± 5.2	54.3 ± 3.9	11.3 ± 2.6	4.8
UT-223-3-2	10.9 ± 0.7	3.1 ± 0.3	17.1 ± 1.4	56.7 ± 1.5	12.2 ± 1.9	4.6
UT-385-4-4	11.6 ± 0.4	3.1 ± 0.3	16.1 ± 1.9	56.3 ± 1.1	12.9 ± 1.7	4.4
UT-475-4-4	11.5 ± 0.8	3.3 ± 0.3	17.8 ± 3.2	55.4 ± 2.8	12.1 ± 2.0	4.6
UT-480-3-2	10.7 ± 0.9	3.4 ± 0.3	17.4 ± 2.1	56.5 ± 1.7	11.9 ± 2.1	4.7
Mean	11.5	3.3	17.5	56.3	11.5	4.9
Pungsannamul	11.3 ± 0.8	3.0 ± 0.2	27.7 ± 10.4	49.9 ± 8.2	8.1 ± 2.8	6.2
Uram	10.6 ± 0.7	3.5 ± 0.5	30.9 ± 13.2	47.2 ± 10.5	7.9 ± 3.0	6.0
Williams 82	10.5 ± 1.1	3.5 ± 0.4	21.0 ± 3.7	56.1 ± 2.9	8.9 ± 2.3	6.3

Table 4. *Cont.*

Genotype	Palmitic Acid	Stearic Acid	Oleic Acid	Linoleic Acid (ω-6)	α-linolenic Acid(ω-3)	Ratio of ω-6 and ω-3
	Mean (%) ± Standard Deviation					
Mean	10.8	3.3	26.5	51.0	8.3	6.2
Overall mean	11.3	3.3	19.3	55.3	10.8	5.1
LSD (5%) [a)]	0.3	0.2	1.6	1.3	0.6	

[a)] LSD is the least square difference and is calculated at 5 % level of significance.

The means of the 18:3 concentration in the soybean genotypes across the eight environments in the three Asian countries are listed in Table 5. The soybean genotypes grown in Vietnam, 2019 (E4), produced the least 18:3 concentration (7.9%), whereas those grown on 19 June, in Gunwi, Korea (E6), had the highest mean 18:3 concentration (12.9%). The mean concentration of 18:3 of soybean genotypes was higher when they were planted in June rather than May in Gunwi (12.5% in E5 and 12.9% in E6, respectively) and in Gwangju (7.9% in E7 and 12.2% in E8, respectively), Korea. The highest 18:3 concentration across the eight environments was the UT-385-4-4 line, ranging from 10.6% to 14.8%, with an average 12.9%, followed by UT-223-3-2, UT-475-4-4, and UT-480-3-2 (Tables 5 and 6). Conversely, check cultivars, including Pungsannamul, Uram, and Williams 82, had lower mean of 18:3 concentration of 8.3%, 8.2%, and 8.9%, respectively, across the eight environments, ranging from 3.4% to 12.5%, 3.6% to 12.9%, and 6.6% to 13.1%, respectively.

Table 5. Mean α-linolenic acid concentration for twelve soybean genotypes with elevated α-linolenic acid concentration and three soybean cultivars with normal α-linolenic acid grown in eight environments over two years.

Genotype	α-linolenic Acid (%)								Mean ± SE
	E1 [a)]	E2	E3	E4	E5	E6	E7	E8	
PT-65-4	10.7	9.9	8.7	7.8	.	12.6	10.4	11.7	10.3 ± 3.9
PT-98-1-4	11.5	9.3	10.1	10.1	12.4	14.6	11.7	11.7	11.4 ± 4.0
PT-100-3	11.7	10.1	9.5	8.7	.	12.2	11.0	11.2	10.6 ± 4.0
PT-1190-2	12.1	9.9	10.6	5.4	13.5	13.7	11.7	12.5	11.2 ± 3.9
PT-1133-4-1	11.9	9.9	8.9	8.1	11.9	11.8	11.4	12.0	10.7 ± 3.8
UT-46-3-3	14.2	10.2	11.2	9.8	9.5	11.4	8.3	14.0	11.1 ± 3.9
UT-124-3-4	11.5	10.1	8.9	8.0	13.5	14.3	13.4	13.5	11.6 ± 4.1
UT-223-1-1	13.2	10.8	9.1	6.0	12.7	13.1	12.9	12.8	11.3 ± 4.0
UT-223-3-2	12.5	10.8	10.5	9.8	.	14.5	14.5	12.8	12.2 ± 4.6
UT-385-4-4	13.5	11.2	10.9	10.6	14.1	13.8	14.8	14.3	12.9 ± 4.6
UT-475-4-4	13.2	9.8	9.4	10.0	14.1	13.6	13.7	13.3	12.1 ± 4.3
UT-480-3-2	13.3	10.4	10.4	8.2	13.5	14.0	13.1	13.4	12.0 ± 4.3
Pungsannamul	8.2	8.2	5.9	3.4	12.5	11.6	8.3	8.7	8.3 ± 2.9
Uram	8.5	4.7	6.2	3.6	12.9	9.6	9.3	10.6	8.2 ± 2.9
Williams 82	8.2	6.6	6.8	9.2	9.8	13.1	7.9	9.9	8.9 ± 3.2
Mean	11.6	9.5	9.1	7.9	12.5	12.9	11.5	12.2	10.9
LSD (5%) [b)]	1.9	2.0	0.7	0.5	2.5	2.6	2.2	1.6	
Temperature (°C) [c)]	27.5	30.2	32.6	33.7	25.3	23.0	25.7	22.6	

[a)] Locations, years, and planting dates over 8 different environments (E1, Vietnam in 2018; E2, Vietnam in 2019; E3, Laos in 2018; E4, Laos in 2019; E5, Gunwi, South Korea, 22 May 2018; E6, Gunwi, South Korea, 19 Jun 2018; E7, Gwangju, South Korea, 29 May 2018; E8, Gwangju, South Korea, 29 Jun 2018). [b)] LSD is the least square difference and is calculated at 5 % level of significance. [c)] Average temperatures during the last 30 days of the reproductive period in E1, E2, E3, and E4, and an average temperature of September in E5 and E7, and an average temperature of mid-September to mid-October in E6 and E8.

The stability parameters, such as the range of average 18:3 concentration, CV, and stability coefficient (b_E) for mean 18:3 concentration of soybean genotypes, are listed in Table 6. The lower the range and CV values, the more stable the concentrations are across environments in which the

soybean genotypes are produced. Range values of PT-1190-2 (9.3) and UT-223-1-1 (8.3) were close to those of the three check cultivars, indicating that these five soybean lines were less stable when compared with other genotypes. PT-100-3 had the least range (3.5) in the 18:3 concentration across the environments studied.

Table 6. Stability parameters: range, coefficient of variation (CV), environmental stability coefficient (b_E), and coefficient of determination (r^2) for mean α-linolenic acid concentration of twelve elevated α-linolenic acid genotypes and three genotypes with normal α-linolenic acid concentration in eight different environments.

Genotype	Mean of α-linolenic Acid		Range of α-linolenic Acid		CV		Stability Coefficients (b_E) [a]				Mean Rank [b]
	%	Rank	%	Rank	%	Rank	b_E	Rank	P	r^2	
PT-65-4	10.3	12	5.4	5	16.1	6	−0.9	8	0.001	0.9	6
PT-98-1-4	11.6	5	5.3	4	13.5	3	−0.8	4	0.007	0.7	3
PT-100-3	10.5	11	3.5	1	11.7	1	−0.7	2	<0.001	0.9	1
PT-1190-2	11.3	7	9.3	14	24.0	12	−1.4	13	0.001	0.9	13
PT-1133-4-1	10.7	10	4.2	2	14.7	4	−0.8	5	<0.001	0.9	4
UT-46-3-3	11.2	9	6.4	9	18.5	9	−0.3	1	0.511	0.1	9
UT-124-3-4	11.5	6	7.0	10	20.5	10	−1.3	11	<0.001	0.9	10
UT-223-1-1	11.3	8	8.3	12	22.7	11	−1.3	12	0.001	0.8	11
UT-223-3-2	12.2	2	5.7	6	15.4	5	−0.9	7	0.004	0.8	5
UT-385-4-4	12.9	1	5.2	3	13.3	2	−0.9	6	0.002	0.8	2
UT-475-4-4	12.1	3	5.9	7	16.4	7	−1.0	9	0.001	0.9	7
UT-480-3-2	11.9	4	6.2	8	17.7	8	−1.1	10	<0.001	1.0	8
Pungsannamul	8.1	14	9.2	13	35.4	14	−1.4	14	0.003	0.8	14
Uram	7.9	15	9.5	15	37.7	15	−1.6	15	0.001	0.8	15
Williams 82	8.9	13	7.8	11	25.4	13	−0.7	3	0.106	0.4	12

[a] Stability coefficients for mean α-linolenic acid concentration of genotypes at each environment regressed on the environmental index. [b] Mean rank was based on the three stability parameters such as range, coefficients of variation (CV), and stability coefficient.

The CV trends for the 18:3 concentration of the soybean genotypes were slightly different from range trends (Table 6). The check cultivars had higher CV values, 35.4% for Pungsannamul, 37.7% for Uram, and 25.4% for Williams 82, when compared with the CV values of the EALA lines. PT-100-3 was the most stable line based on the CV value (11.7%) among fifteen soybean genotypes across eight environments, followed by UT-385-4-4 (13.3%), PT-98-1-4 (13.5%), and PT1133-4-1 (14.7%).

The stability coefficients (b_E) of each of the soybean genotypes for the 18:3 concentration represent variations in environmental stability across eight environments in three Asian countries (Table 6). The soybean genotypes with b_E values closest to zero are more stable across the eight environments. The stability coefficient values for 18:3 concentration ranged from −0.3 to −1.6. The UT-46-3-3 genotype was the most stable, with highest stability coefficient ($b_E = -0.3$, $p = 0.511$, $r^2 = 0.1$) among the soybean genotypes tested, followed by the PT-100-3 genotype ($b_E = -0.7$, $p < 0.001$, $r^2 = 0.9$). Notably, the 18:3 concentration of Williams 82 was stable across the environments based on the stability coefficient ($b_E = -0.7$, $p < 0.106$, $r^2 = 0.4$), when compared with the range and CV values. A GGE plot was visualized to select the best soybean lines in specific environments (Figure 1). The perpendiculars divide the biplot into the six sectors. Soybean genotypes were in the polygon, and the best soybean genotypes for each sector were located on the vertices of polygon. Soybean genotype UT-385-4-4 was the best soybean line to produce 18:3 concentration in E1 (Vietnam in 2018), E2 (Vietnam in 2019), E3 (Laos in 2018), E6 (Gunwi, South Korea, 19 Jun 2018), E7 (Gwangju, South Korea, 29 May 2018), and E8 (Gwangju, South Korea, 29 Jun 2018). None of these environments were in the sectors with Uram and Pungsannamul as the vertices, meaning that these cultivars were not the best in any of the environments.

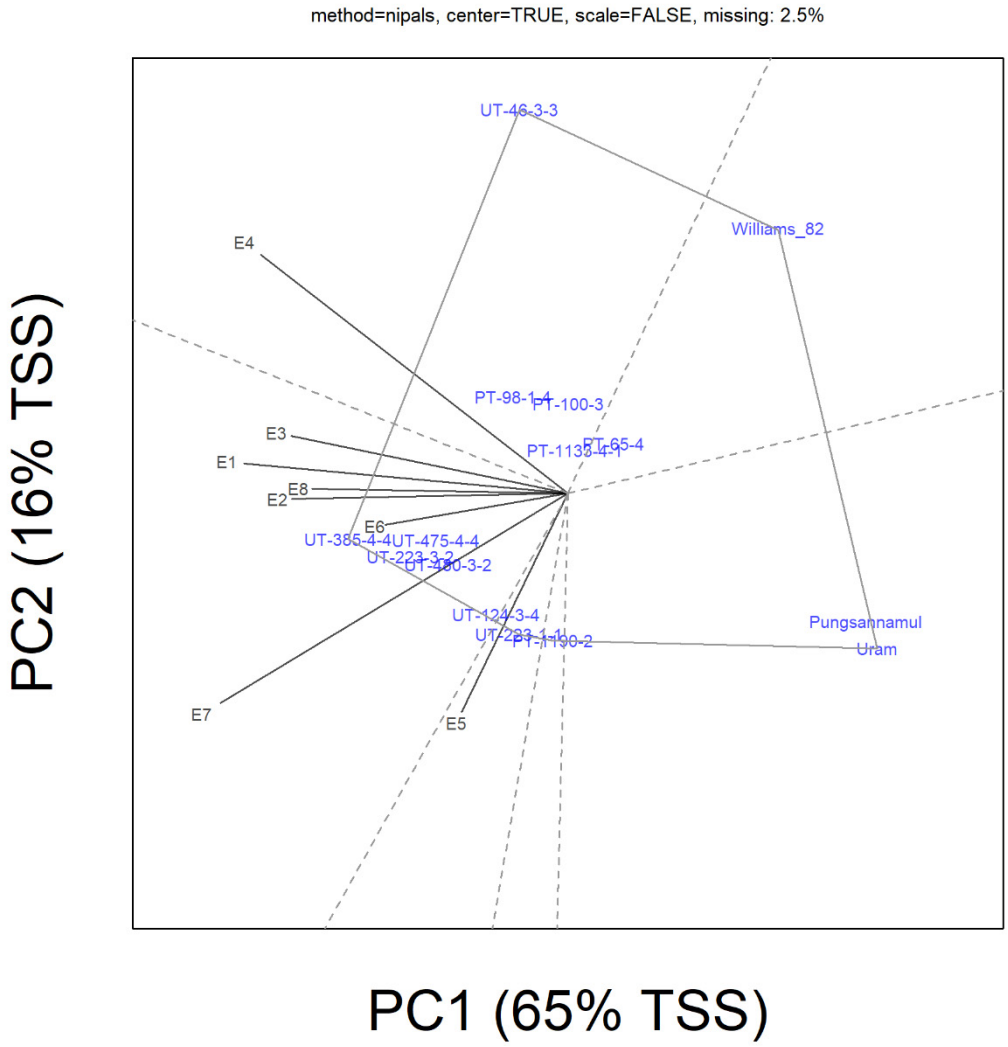

Figure 1. The which-won-where of the genotype plus genotype by environment (GGE) biplot to explain the accumulation of linolenic acid in multiple environments. PC1 and PC3 are the first and second principal components, respectively. E1, Vietnam in 2018; E2, Vietnam in 2019; E3, Laos in 2018; E4, Laos in 2019; E5, Gunwi, South Korea, 22 May 2018; E6, Gunwi, South Korea, 19 Jun 2018; E7, Gwangju, South Korea, 29 May 2018; E8, Gwangju, South Korea, 29 Jun 2018; TSS, total sum of squares.

Mean ranks were determined based on the three stability parameters, including ranges of 18:3 concentration, CVs, and stability coefficient values listed in Table 6. The PT100-3 soybean genotype had the most stable for 18:3 concentration, with an average concentration of 10.5% across the eight study environments, followed by UT-385-4-4, which was ranked second based on the mean rank, with the highest mean value of the 18:3 concentration among the soybean genotypes. The 18:3 concentration in the check cultivars was less stable when compared with the EALA soybean genotypes based on mean rank, excluding PT-1190-2.

4. Discussion

As soybean is one of the most economically important oil crops, most studies have focused on the reduction of 18:3 concentration to improve oxidative stability. However, in many Asian countries, soybean has been consumed directly in both fermented and non-fermented forms. 18:3 (ω-3) is one of the most important essential fatty acids in human diets, and is the precursor of EPA and DHA, which prevent several diseases such as inflammation, cardiovascular disease, and Alzheimer's disease, as

well as promoting fetal development [15,16]. Therefore, it is important to increase 18:3 concentration in soybean breeding programs.

Wild soybean can be exploited as a genetic resource to develop soybean lines with high 18:3 concentration [20,22]. However, exploiting wild soybeans in breeding programs is challenging due to their poor agronomic traits such as small seeds, vine growth habit, seed shattering, and seed hardness. In the present study, we developed soybean lines with EALA concentration from a wild soybean, PI 483463, with 15.4% 18:3 concentration [20]. As soybean genotypes were backcrossed with three cultivated soybeans with normal 18:3 concentration, the genomic constitution of EALA soybean genotypes genomes were 87.5% similar to those of the cultivated soybean genomes. Therefore, phenotypes such as erect type, yellow seed coat, less lodging, leaf size, and seed size of the soybean genotypes containing desaturase alleles that increase 18:3 concentration from a source of wild soybean were close to the cultivated soybeans.

We evaluated how environmental and genetic factors influence the accumulation of different fatty acid profiles in EALA soybean lines across eight environments in three Asian countries. Our results revealed that the environment was the key factor influencing different fatty acid profiles (Figure 1, Table 2). In addition, genotype and genotype × environment interactions influenced fatty acid profiles, based on the ANOVA results (Table 2) [20,21,42,43]. We observed that the environment was the most critical factor influencing 18:3 concentration in soybean oil, accounting for 73.7% of the variation, which is consistent with the findings of Hou et al. [28]. However, soybean lines from high 18:3 concentration alleles of wild soybean had higher levels of 18:3 when compared with cultivated soybeans across nine different environments [20].

Generally, in soybeans, unsaturated fatty acids are less stable when compared with saturated fatty acids. Higher temperatures during pod-filling periods are associated with decreased 18:2 and 18:3 concentrations and increased 18:1 concentration in soybean seed oil [21,27,29,32,33]. We observed that soybean genotypes grown under high temperatures in the pod-filing periods had lower amounts of 18:3 concentration (Table 5). In addition, planting dates influenced the accumulation of fatty acid profiles. According to Wilcox and Cavins [44], soybean genotypes planted late produced higher 18:3 concentration than early planted soybean genotypes. The results of the presented study also indicated that planting dates influenced the accumulation of 18:3 concentration for most of the soybean genotypes in two locations (Table 5).

The intake of either fatty acids with lower ω-6/ω-3 ratios or the intake of fatty acids with high ω-3 is desirable for human health [17–19]. Recently, Kulkarni et al. [24] suggested that the combination of the microsomal delta-12 fatty acid desaturase 2 genes, with alleles of wild soybean to elevate 18:3 concentration, could reduce the ω-6/ω-3 fatty acid ratios to the 1.7 to 2.5 range, considering cultivated soybean has an approximate ω-6/ω-3 ratio of 6.0, while increasing both 18:0 and 18:3 concentrations and decreasing 18:2 concentration.

Asekoba et al. [20] reported that the ω-6/ω-3 ratios of three RILs from a single cross of Hutcheson and wild soybean, PI 483463, were 3.7, 3.9, and 4.1, whereas that of a check cultivar, Williams 82, was 6.9. Similarly, in the present study, a soybean genotype, UT-385-4-4, had the highest 18:3 concentration in three Asian countries and the lowest ω-6/ω-3 ratio (4.4). Based on the three stability parameters explored in the present study, PT-100-3 was the most stable producer of 18:3 concentration across eight environments with a mean concentration of 10.5% (Table 6). However, UT-385-4-4 produced the highest 18:3 concentration (12.9%) and was more stable in the study than all other soybean genotypes, excluding PT-100-3. In addition, UT-385-4-4 was the best soybean genotype across six environments based on the result of GGE biplot (Figure 1). To develop a novel soybean cultivar with high 18:3 concentration for soybean foods, UT-385-4-4 is a potential genetic resource for exploitation in breeding programs.

One of our earlier studies observed that Korea was one of the centers of origin of domesticated soybean with relatively high 18:3 concentration evaluated in different environments based on wild soybean collections in the country [22,45]. The results indicated that two wild soybeans had much higher 18:3 concentration and more stable fatty acid profiles than a check cultivar, with means in the

18.5% to 19.1% range. Dhakal et al. [21] conducted a stability study in Korea with EALA cultivated plant introductions whose 18:3 concentration were available in the USDA soybean germplasm collections, as reported in germplasm resources information network, ranging from 8.5% to 15.5%. However, the highest value in plant introductions was 10.7%. Pantalone et al. [25] suggested that the accumulation of high concentration of 18:3 in wild soybean was due to a different set of desaturate alleles controlling 18:3 in cultivated soybean. Through QTL analyses, six novel QTLs were linked to the accumulation of 18:3 concentration in wild soybean, suggesting that the QTLs are different from the ones influencing 18:3 concentration in cultivated soybean [23]. The accumulation of 18:3 concentration in cultivated and wild soybean is still unknown. Thus, further genetic, breeding, molecular biology, and genomic investigations are required to enhance our understanding of the factors influencing 18:3 concentration in both cultivated and wild soybean.

Generally, dietary ω-3 fatty acids known as EPA and DHA are obtained from fish and fish products globally. However, as fish catch gradually decreases due to environmental pollution, climate change, indiscriminate fishing, and increased poaching, fish and fish products are likely to be inadequate sources of ω-3 in many countries. Vegetable oils, such as canola and soybean oil, are major sources of 18:3, shorter chain ω-3 fatty acids. Among oil crops, flaxseed, perilla, and English walnut are rich in 18:3. Plant-derived ω-3 can be potential alternative sources of ω-3 fatty acids for populations living in developing tropical and subtropical countries, with high risks of inadequate ω-3 intake.

The development of novel soybean cultivars with high protein and elevated 18:3 concentration could enhance human health in populations consuming soybean foods. As the present study was conducted in two subtropical regions—Vietnam and Laos—EALA lines produced more stable 18:3 concentration than those of check cultivars. Therefore, the EALA lines could be exploited as genetic resources in subtropical and tropical regions for the development of novel soybean cultivars with high levels of 18:3 concentration, which could be sources of ω-3 fatty acids.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0472/10/3/70/s1, Figure S1: Comparison of relative concentrations of mean squares of factors on each fatty acid for all soybean genotypes over different environments. *** Significant at the 0.05 probability level.

Author Contributions: J.-D.L. conceived and designed the study. H.J. analyzed the results and drafted the manuscript. M.K. and L.A. determined the fatty acid profiles. R.T., D.J., D.T.L., S.P., B.-K.H., and S.K. organized the fieldwork, and collected the samples from each environment. J.T.S. and J.-D.L. reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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