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ORIGINAL PAPER

Identification of quantitative trait loci controlling linolenic acid concentration in PI483463 (*Glycine soja*)

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Abstract
Key message **The QTLs controlling alpha-linolenic acid concentration from wild soybean were mapped on nine soybean chromosomes with various phenotypic variations. New QTLs for alpha-linolenic acid were detected in wild soybean**.
Abstract Alpha-linolenic acid (ALA) is a polyunsaturated fatty acid desired in human and animal diets. Some wild soybean (*Glycine soja*) genotypes are high in ALA. The objective of this study was to identify quantitative trait loci (QTLs) controlling ALA concentration in a wild soybean accession, PI483463. In total, 188 recombinant inbred lines of $F_{5:6}$, $F_{5:7}$, and $F_{5:8}$ generations derived from a cross of wild soybean PI483463 (~15 % ALA) and cultivar Hutcheson (~9 % ALA) were planted in four environments. Harvested seeds were used to measure fatty acid concentration. Single nucleotide polymorphism markers of the universal soybean linkage panel (USLP 1.0) and simple sequence repeat markers were used for molecular genotyping. Nine putative QTLs were identified that controlled ALA concentration by model-based composite interval mapping and mapped to different soybean chromosomes. The QTLs detected in four environments explained 2.4–7.9 % of the total phenotypic variation (PV). Five QTLs, *qALA5_3*, *qALA6_1*, *qALA14_1, qALA15_1*, and *qALA17_1*, located on chromosomes 5, 6, 14, 15, and 17 were identified by model-based composite interval mapping and composite interval mapping in two individual environments. Among them, *qALA6_1* showed the highest contribution to the PV with 10.0–10.2 % in two environments. The total detected QTLs for additive and epistatic effects explained 52.4 % of the PV for ALA concentration. These findings will provide useful information for understanding genetic structure and marker-assisted breeding programs to increase ALA concentration in seeds derived from wild soybean PI483463.

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Introduction

In soybean [*Glycine max* (L.) Merr.], linolenic acid is an important fatty acid affecting seed oil quality. Soybean oil from current commercial cultivars typically contains approximately 8–10 % linolenic acid (Wilson 2004). However, alpha-linolenic acids (ALAs, ω-3) are highly susceptible to oxidation, which causes undesirable odors and a shorter shelf life in soybean oil (Dutton et al. 1951; Smouse

1979). Therefore, efforts have been made to develop soybean lines with low linolenic acid concentration. The natural or artificial mutations in three soybean microsomal omega-3 fatty acid desaturase genes, *GmFAD3A*, *GmFAD3B*, and *GmFAD3C*, reduced the ALA concentration below 4 % (Anai et al. 2005; Bilyeu et al. 2003, 2005; Chappell and Bilyeu 2006, 2007). Pyramiding the three mutant alleles allowed the development of new soybean lines containing 1 % linolenic acid (Bilyeu et al. 2006, 2011).

However, ALA, an omega-3 fatty acid, is an essential fatty acid in human and animal diets and is a precursor of long-chain polyunsaturated fatty acids (LC-PUFAs), eicosapentaenoic acid (EPA; 20:5), and docosahexaenoic acid (DHA; 22:6). Higher intake of LC-PUFA was associated with reduced risks of cardiovascular disease (Calder 2004; Jakobsen et al. 2009; Kris-Etherton et al. 2002) and inflammatory disease (Calder 2003). In addition, it had beneficial effects on the early development of visual and brain systems during pregnancy and early postnatal life (Alessandri et al. 2004; Jeffrey et al. 2001). A higher dietary intake of ALA from vegetable oil significantly reduced the risk of clinical depression and breast cancer in women (Lucas et al. 2011; Thiébaut et al. 2009).

The dietary ratio of ω-6/ω-3 fatty acids also played an important role in moderating T cell mediated immune responses in prostate cancer (Han et al. 2012; Williams et al. 2011). Diets high in linoleic acid (LA, ω-6) concentration reduced the nutritionally positive effects of the health-beneficial omega-3 fatty acids in tissue (Blasbalg et al. 2011; Clark et al. 1992; Friesen and Innis 2010). Therefore, a lower ratio of LA–ALA (4:1 or less versus ~6–7:1, as found in commercial soybean seed) or a high ω-3 intake was important to suppress illnesses. A ratio of LA–ALA of 4:1 was found to prevent cardiovascular diseases; a ratio of 2.5:1 inhibited growth of colon cancer cells; a ratio of 2–3:1 reduced rheumatoid and inflammatory arthritis, and of a ratio of 5:1 was associated with a reduction in asthma (Simopoulos 2008). An ideal diet consists of ω-6:ω-3 fatty acid ratios that range from 1:1 to 4:1 (Mattson and Grundy 1985; Simopoulos 2002).

There have been few breeding and genetic studies focused on increasing high linolenic acid concentration in soybean. Soybean mutant lines with elevated linolenic acid (12.6–12.9 %) were developed from cultivated soybean with chemical or X-ray mutagenesis (Hudson 2012; Rahman et al. 1994). Recently, Dhakal et al. (2013) reported that the elevated linolenic acid in 18 (*G. max*) soybean accessions from the USDA Soybean Germplasm Collection varied from 8.5 to 15.5 % in linolenic acid concentrations. These 18 *G. max* accessions averaged only 6.5–10.7 % linolenic acid concentrations over 10 environments. This suggests that there was a lack of *Glycine max* germplasm sources to significantly elevate linolenic acid in the soybean seed oil.

However, wild soybean (*Glycine soja* Sieb and Zucc.) accessions contained as much as 23 % linolenic acid (Pantalone et al. 1997a; Shibata et al. 2008). In *G. max* × *G. soja* populations, Shibata et al. (2008) identified four QTLs that controlled linolenic acid concentration in a wild soybean accession "Hidaka 4". Among these, one QTL was linked to a Satt384 marker on chromosome (Chr) 15 and explained 36.9 % of the total phenotypic variation for linolenic acid concentration. The average linolenic acid concentration of the lines derived from the Hidaka 4 genotype was 15.3 %, and that derived from cultivated soybean genotypes was 12.7 %. In *G. max* × *G. soja* populations, the higher linolenic acid lines had lower seed mass and lipid concentration (Pantalone et al. 1997b; Shibata et al. 2008).

The purpose of this study was to genetically map QTLs controlling elevated linolenic acid concentration from PI483463 using a population of 188 recombinant inbred lines (RILs) developed from an interspecific cross between wild soybean accession PI483463 (*G. soja*), with ~15 % ALA concentration, and the cultivated soybean Hutcheson (*G. max*), with ~9 % ALA concentration.

Materials and methods

Population development and field trials

A recombinant inbred line (RIL) population, developed from a cross of PI483463 (*G. soja*) and a cultivated soybean cultivar, Hutcheson, was utilized for a genetic mapping study (Ha et al. 2013). The linolenic acid concentration differed between the two parents. PI483463 had approximately 15 % linolenic acid, while the cv. Hutcheson had approximately 9 % linolenic acid (Asekova et al. 2014). The initial cross was made in a greenhouse during the winter of 2005–2006 at the University of Missouri-Delta Research Center (UMDRC), Portageville, MO, USA. F_1 seeds were planted at the Lee Farm, UMDRC during the summer of 2006. The single-hill procedure was applied to advance generations of this population from 2006 to winter of 2007–2008 in a greenhouse (winter) and soybean field (summer). In short, F_2 seeds were planted in a greenhouse during the winter of 2006–2007 to produce $F_{2:3}$ seeds. Two to three $F_{2:3}$ seeds from each F_2 plant were planted in a hill in the field during the summer of 2007 to produce $F_{3:4}$ seeds. Subsequently, 2–3 $F_{3:4}$ seeds from each F_3 plant from each hill were planted in a greenhouse during the winter of 2007–2008 to produce $F_{4:5}$ seeds. One hundred ninety F_5 plants each tracing to a single F_2 plant were planted in the field in 10 seed hill plots at the Lee Farm, UMDRC, in the summer of 2008. Each hill was thinned to

a single F_5 plant and seeds of 188 $F_{5:6}$ RILs were harvested in the fall of 2008. Fatty acid concentration was evaluated for the parents and the 188 RILs in four growing environments. Environments constituted plantings in different years and locations with slightly modified planting dates and plant densities. The $F_{5:6}$ and $F_{6:7}$ RILs were planted at Lee Farm (Portageville, 36°44′N, MO, USA) on May 22, 2008, and on June 19, 2009, respectively. $F_{5:8}$ RILs were planted at the Affiliated Experiment and Practice Fields of Kyungpook National University (Gunwi, 36°14′N, Republic of Korea) on May 14, 2010, and May 25, 2012. In the research conducted at the Lee Farm and Gunwi, soybeans were planted in hills with between-row spacings of 76 and 85 cm, respectively, and intra-row spacings for the hill plots were 61 and 95 cm, respectively. A randomized complete block design (RCBD) with two replications was used in each environment. Approximately 10 seeds of each RIL were planted in each hill in all environments. At maturity, plants of each hill plot were bulked and threshed using a small bundle thresher.

Analysis of fatty acid composition

The fatty acid profile (relative percentage of total fatty acids) for each RIL within each environment was determined for each plot as described in previous studies with minor modifications (Lee et al. 2009, 2012). For each environment, 10 randomly selected seeds from each plot and genotype were placed in a paper envelope and were manually crushed. Small samples were taken from each envelope and placed in test tubes for oil extraction. The oil was extracted by placing crushed seeds in a 5 mL solution of chloroform: hexane: methanol (8:5:2, v/v/v) overnight. Derivatization was performed by transferring 100 µl of the extract to vials and adding 75 µl of the methylating reagent (0.25 M methanolic sodium methoxide: petroleum ether: ethyl ether [1:5:2, v/v/v]). Hexane was added to bring samples to approximately 1 mL. An Agilent (Palo Alto, CA) Series 7890 capillary gas chromatograph fitted with a flame ionization detector (FID) with an AT-Silar capillary column (Altech Associates, Deerfield, IL) was used to analyze the fatty acid concentration. The temperature of the oven, injector, and detector was set at 210, 250, and 230 °C, respectively. Standard fatty acid mixtures (Animal and Vegetable Oil Reference Mixture 6, AOACS) were used as calibration reference standards.

Statistical analysis

The statistical analyses, including RIL frequency distribution, the mean of the RIL population, the coefficient of variation (CV), the broad sense heritability value (h^2) and the analysis of variance (ANOVA) were conducted using the SAS program (SAS Institute Inc. 2004). The heritability in a single environment was estimated using $h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2/r)$, where h^2 is heritability, σ_g^2 is the genotypic variance component for linolenic acid concentration per plot among RILs, σ_e^2 is the error variance, and r is the number of replications for the trait (Xing et al. 2012). The heritability value (h^2) over years was calculated from $h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_{gy}^2/n + \sigma_e^2/nr)$, where σ_g^2, σ_{gy}^2, and σ_e^2 are the genotype, genotype × environments interaction, and error components of variance, respectively, n is the number of environments, and r is the number of replications (Hanson et al. 1956). All parameters were estimated from the expected mean squares in the ANOVA.

Single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) analysis

Five to seven seeds of each $F_{5:6}$ RIL were planted in a greenhouse. Young trifoliate leaves of V2 seedlings were collected for DNA extraction using the automated Autogen 960 system and the CTAB protocol of the manufacturer (AutoGen Inc., Holliston, MA, USA) with minor modifications (Ha et al. 2013; Vuong et al. 2010).

The universal soybean linkage panel 1.0 (USLP 1.0) with 1,536 SNP loci, developed for the soybean genome (Hyten et al. 2008) and mapped onto the integrated molecular genetic linkage map, was employed to genotype the $F_{5:6}$ RIL mapping population (Vuong et al. 2010) using the Illumina GoldenGate assay (Fan et al. 2006; Hyten et al. 2010). In brief, genomic DNA samples of RILs were activated by biotinylation. Following PCR amplification with three oligonucleotide sequences (two allele-specific oligos and one locus-specific oligo) and oligonucleotide hybridization, washing was conducted to remove excess and mishybridized oligos. The resulting double-stranded PCR products were immobilized onto paramagnetic particles, followed by washing and denaturing. The released single-stranded DNAs were then hybridized to their complementary bead type. Array imaging was performed using the Illumina BeadStation (Illumina, San Diego, CA, USA) to generate intensity data files. The allele calling for each SNP locus was subsequently performed using the BeadStudio 3.0 program (Illumina, San Diego, CA, USA). The clusters of homozygous and heterozygous genotypes for each SNP locus were carefully checked for polymorphic SNP markers and were then used to construct a genetic map as previously described (Vuong et al. 2010).

In addition to SNP markers of the USLP 1.0 panel, a subset of SSR markers on Chrs 5, 6, 14, and 15 were selected from a soybean SSR database (BARCSOYSSR_1.0) and RILs were genotyped as described by Vuong et al. (2010). Sixteen polymorphic SSR markers on Chrs 5, 6, 14, and 15 were integrated into a SNPs-based genetic linkage map to

Table 1 Summary of statistics on the alpha-linolenic acid (ALA) concentration of the parental lines and 188 recombinant inbred lines (RILs) developed from a cross between PI483463 and Hutcheson for their ALA concentration, which was evaluated at the University of Missouri-Delta Research Center, MO, USA, in 2008 and 2009 and at Gunwi, Korea, in 2010 and 2012

Year	ALA (%)						CV (%)[a]	F[b]	h^2 (%)[c]
	Parent		RILs						
	Hutcheson	PI483463	Mean	Min	Max	SD			
2008	8.7	15.9	11.6	7.9	14.8	1.15	3.9	12.6**	92.1
2009	9.7	17.1	12.9	9.0	16.3	1.26	3.3	17.2**	94.2
2010	8.9	15.7	11.4	8.0	15.8	1.40	7.3	7.4**	86.5
2012	8.9	17.4	11.3	7.9	16.3	1.42	5.6	10.5**	90.5
Combined	9.1	16.5	11.8	8.6	14.1	0.90	5.1	18.9**	61.2

** $P < 0.001$

[a] Coefficient of variation

[b] F value is for determining significance among lines

[c] Heritability value was obtained from ANOVA

reduce the gaps between mapped SNP markers that were associated with linolenic acid-related QTLs.

QTL analysis

Linkage analysis was performed with the computer program MapManager QTX b20 (Manly et al. 2001) using the Kosambi mapping function (Kosambi 1944), with a minimum LOD score of 3.0 and a maximum genetic distance of 50 cM. For identification of candidate QTL regions for linolenic acid concentration, QTL mapping was performed with composite interval mapping (CIM) executed with WinQTL Cartographer 2.5 (Wang et al. 2011) and a mixed model-based composite interval mapping (MCIM) procedure of QTLNetwork V2.0 (Yang et al. 2008). QTL detection was first undertaken using the CIM for each environment followed by conducting MCIM across environments to confirm the CIM results.

The main additive effect of the QTLs and their genetic effects were detected by composite interval mapping using the WinQTL Cartographer 2.5 (Wang et al. 2011). In each environment, CIM was performed using Model 6 after scanning the genetic map with the LOD significance threshold determined using a 1,000 permutation tests with a walk speed of 1 cM and a significance level of 0.05. The QTLNetwork V2.0 was used to estimate additive QTLs, epistatic QTL pairs, additive QTL × year, and epistatic QTL pair × year interactions across environments. The general model of MCIM incorporated the fixed terms of additive effects (A) and additive × additive epistatic effects (AA), as well as random terms for environment, additive × environment interaction, and epistasis × environment interaction effects. Significant QTLs were defined by an F-statistic profile obtained from a 2D genome scan procedure. The critical F values were calculated by permutation tests of 1,000 times at a genome-wide 0.05 significance level with a forward and backward selection step. Finally, QTL peak regions that exceeded the threshold F value were declared significant, while significant epistatic effects and QTL × environment interaction effects were estimated based on P values ($P < 0.05$).

The chromosomes with LOD plots were created using the MapChart 2.2 program (Voorrips 2002) based on the outputs from QTL Cartographer. The nomenclature for detected QTLs was denoted as *qALA3_1*, where "qALA" represents a QTL for the ALA concentration in soybean and *3_1* represents the first QTL on Chr 3. Two QTLs with shared marker(s) or with position(s) close (<5 cM apart) to those on the consensus genetic map (Song et al. 2004) were considered to be the same QTL.

Results

Phenotypic variation

There were significant differences between the two parents in each of the four environments (Table 1; Fig. 1). The mean ALA concentrations across environments for the Hutcheson parent and the PI483463 parent were 9.1 and 16.5 %, respectively. The RIL population also showed broad ranges of variation across environments, which ranged from 7.9 to 16.3 %, with a mean of 11.8 %. This RIL population showed significant variation among RILs in each environment during the 4-year study ($P < 0.01$). In particular, the RIL population in the 2009 environment showed a higher mean ALA concentration than the other three environments. The RIL population was normally distributed for all four environments and showed similar distribution patterns across each environment. The coefficient

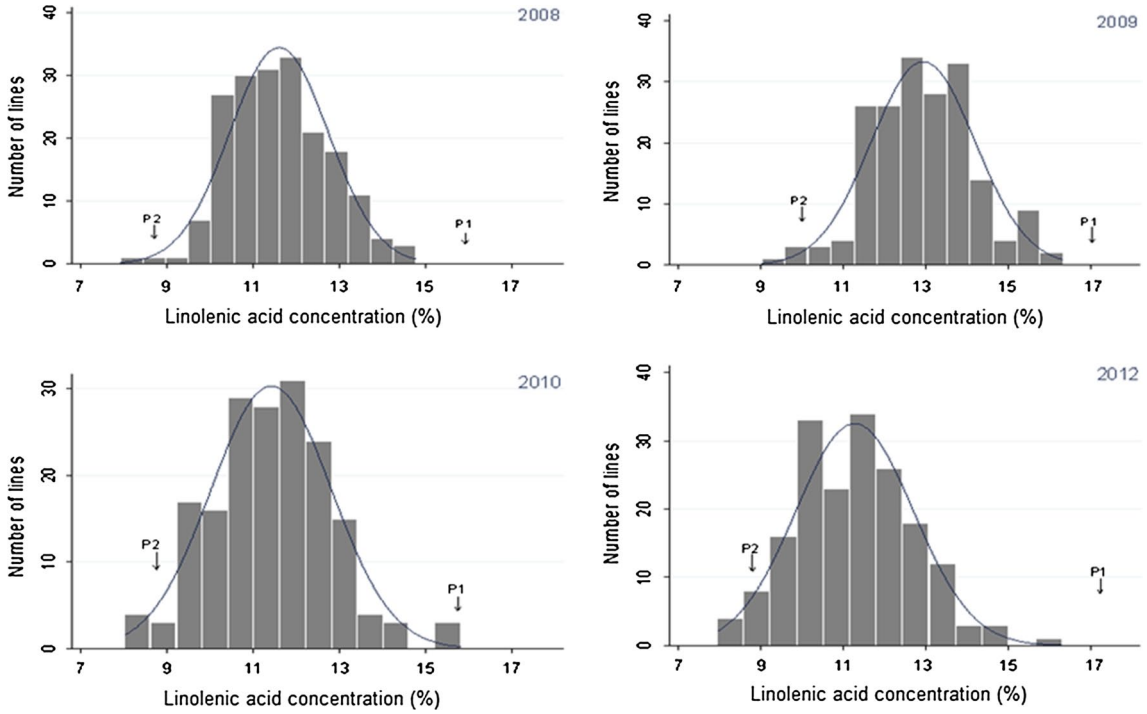

Fig. 1 Distribution of alpha-linolenic acid concentration among 188 recombinant inbred lines (RILs) from a cross between PI483463 (P1) and cv. Hutcheson (P2) evaluated at the University of Missouri-Delta Research Center, MO, USA, in 2008 and 2009 and at Gunwi, Korea, in 2010 and 2012

of variation (CV) for ALA concentration in each year was low and ranged from 3.3 to 7.3 %. The CV at Gunwi, Korea, was greater than that at Portageville, MO, USA, indicating that the relative error variation for ALA concentration in Korea was greater than in the USA. The heritability of ALA concentration was high and ranged from 86.5 to 94.2 % in each of the 4 years (Table 1). This suggested that genetic variation accounts for a major part of the phenotypic variation for ALA concentration in this RIL population. In the ANOVA, the *F* value for the genotype × environment interaction was significant. However, it was less than the *F* value of the genotype (Table 2). Therefore, all data were analyzed across environments for linkage mapping.

Table 2 ANOVA of ALA concentration in RILs of PI483463 × Hutcheson over four environments

Source	DF[a]	MS[b]	*F*[c]
Genotype	187	6.9	18.9**
Environment	3	238.1	657.4**
Genotype × environment	555	2.7	7.4**
Error	745	0.4	

** $P < 0.001$

[a] Degrees of freedom

[b] Mean square

[c] *F* value is for determining significance

QTLs associated with ALA concentration in different environments

A molecular linkage map consisting of 546 SNPs and 16 SSR markers was constructed, covering 2,852 cM across 20 soybean chromosomes with an average interval of 5 cM between adjacent markers. A total of 12 QTLs associated with ALA concentration were detected in at least one of the four environments using CIM analysis based on QTL Cartographer (Table 3; Fig. 2). In 2008, five QTLs on Chrs 5, 11, 14, 15, and 17 were detected, explaining 5.9–15.9 % of the phenotypic variance (PV). In addition, five QTLs on Chrs 5, 6, 14, 15, and 17 were detected in 2009, explaining 7.3–14.3 % of phenotypic variance. However, only three QTLs on Chrs 6, 11, and 12 were detected in 2010, explaining 7.6–10.5 % of the phenotypic variance. In 2012, four QTLs on Chrs 3, 5, 12, and 15 were detected, explaining 6.9–10.5 % of the phenotypic variance.

Among the detected QTLs, *qALA3_1* with a 6.9 % PV was detected only at Gunwi Korea in 2012. The QTL, *qALA5_2* (8.1 % PV) was detected at 92.3 cM of Chr 5 that was 14 cM away from *qALA5_3*. Five QTLs, *qALA5_3*,

Table 3 QTLs associated with ALA concentration using composite interval mapping from 188 RILs derived from a cross of PI483463 × Hutcheson at the University of Missouri-Delta Research Center, MO, USA, in 2008 and 2009 and at Gunwi, Korea, in 2010 and 2012

Year	QTL	Chr	Flanking marker	Position (cM)	LOD[a]	R^2 (%)[b]	A (%)[c]
2008	*qALA5_3*	5	BARC-026129-052~BARC-059049-155	106.4	5.3	8.0	−33
	qALA11_2	11	BARC-042837-084~BARC-050091-093	29.0	3.8	5.9	−29
	qALA14_1	14	BARC-013273-004~SSR14-1370	109.6	8.2	13.8	−43
	qALA15_2	15	BARC-063195-182~BARC-028907-060	42.5	9.6	15.9	−46
	qALA17_1	17	BARC-065705-196~BARC-056107-140	21.3	4.3	7.0	−31
2009	*qALA5_3*	5	BARC-026129-052~BARC-059049-155	106.7	4.9	8.1	−36
	qALA6_1	6	SSR06-657~SSR06-682	85.2	5.4	10.0	−40
	qALA14_1	14	BARC-013273-004~SSR14-1370	113.6	7.0	14.3	−48
	qALA15_1	15	SSR15-0141~BARC-054257-124	27.9	4.0	7.3	−34
	qALA17_1	17	BARC-065705-196~BARC-056107-140	21.3	4.8	8.7	−38
2010	*qALA6_1*	6	SSR06-657~SSR06-682	84.2	5.1	10.2	−46
	qALA11_1	11	BARC-032333-089~BARC-030863-069	12.8	4.4	7.6	−41
	qALA12_2	12	BARC-039403-074~BARC-050003-092	97.7	5.8	10.5	−46
2012	*qALA3_1*	3	BARC-028539-059~BARC-065687-196	111.1	3.8	6.9	−38
	qALA5_2	5	BARC-053443-118~BARC-049091-108	92.3	4.1	8.1	−41
	qALA12_1	12	BARC-029055-060~BARC-018895-030	83.4	5.4	10.5	−47
	qALA15_1	15	SSR15-0141~BARC-054257-124	26.9	5.7	9.5	−45

[a] Logarithm of odds

[b] Phenotypic variation explained by the QTL

[c] Additive effect: negative values indicate that PI483463 contributed the allele for an increase in the trait value

qALA6_1, *qALA14_1*, *qALA15_1*, and *qALA17_1*, were detected in two environments. The QTL *qALA5_3* was positioned at 106 cM of Chr 5 with PVs of 8.0 and 8.1 %, respectively from MO, USA in 2008 and 2009. The QTL, *qALA6_1*, had a PV of 10 % and was detected from the MO, USA location in 2009 and Gunwi, Korea environment in 2010. The QTL, *qALA14_1*, was detected in only US environments and showed the largest PVs, 13.8 and 14.3 % in 2008 and 2009 of all detected QTLs for ALA concentration. The QTL, *qALA15_1*, was detected on Chr 15 from two locations, MO, USA 2009 and Gunwi, Korea 2012 and had PVs of 7.3 and 9.5 %, respectively. Finally, the QTL, *qALA17_1*, was detected on Chr 17 in the two MO, USA environments during 2008 and 2009 and had PVs of 7.0 and 8.0 %, respectively. All the QTLs showed negative additive effects, indicating that the alleles from PI483463 contributed to the elevated ALA concentration in soybean seed.

Based on the QTL identification for ALA concentration across four environments with the QTLNetwork V2.0 program, a total of nine additive QTLs were identified and showed negative effects, meaning the alleles from PI483463 contributed to the ALA concentration (Table 4). Among the nine QTLs five, *qALA5_3*, *qALA6_1*, *qALA14_1*, *qALA15_1*, and *qALA17_1* with additive effects were identified in at least two of the four environments using CIM analysis. Thus, these five QTLs detected from this QTL population are major QTL for ALA concentration. All the QTLs were detected by the MCIC mapping procedure and explained <10 % of the PV. Among them, *qALA12_2* on Chr 12 showed the highest PV (7.9 %) for controlling ALA concentration in soybean.

Epistasis and QTL interaction with environment

Three pairs of epistatic QTLs involving six loci in five chromosomes were identified for ALA concentration and contributed 0.7–2.5 % of PV (Table 5; Fig. 3). Pair 1 was composed of two additive QTLs, *qALA13_1* on Chr 13 and *qALA15_1* on Chr 15 explaining 0.7 % of the PV for ALA concentration. Pair 2 did not display additive effects. Pair 2, involving *qALA4_1* and *qALA5_1,* contributed 2.5 % of the phenotypic variation and explained a negative 25 % of the epistatic effect. Pair 3 with no additive effect, included *qALA4_2* and *qALA11_1* with a positive 25 % epistatic effect and contributed 2.3 % of the phenotypic variation.

In this study, additive QTL × environment and epistatic QTL pair × environment interactions contributed to 0.0–0.5 % of the phenotypic variation in ALA concentration. Thus, these environmental interactions were negligible (Tables 4, 5). Positive epistatic effect QTL pair means that two epistatic loci have different alleles, one from PI483463 and another one from cv. Hutcheson. Negative epistatic effect QTL pair indicates that two epistatic alleles with homozygous alleles from PI483463 could increase ALA concentration in soybean seed.

Genetic structure of ALA concentration

The genetic structure for ALA concentration was analyzed for the dataset over 4 years using MCIM. In Table 6, the total genetic variance proportion is equivalent to the heritability value estimated from the ANOVA. The proportion of additive QTL and epistatic QTL pairs are obtained from the MCIM results, and the difference between total

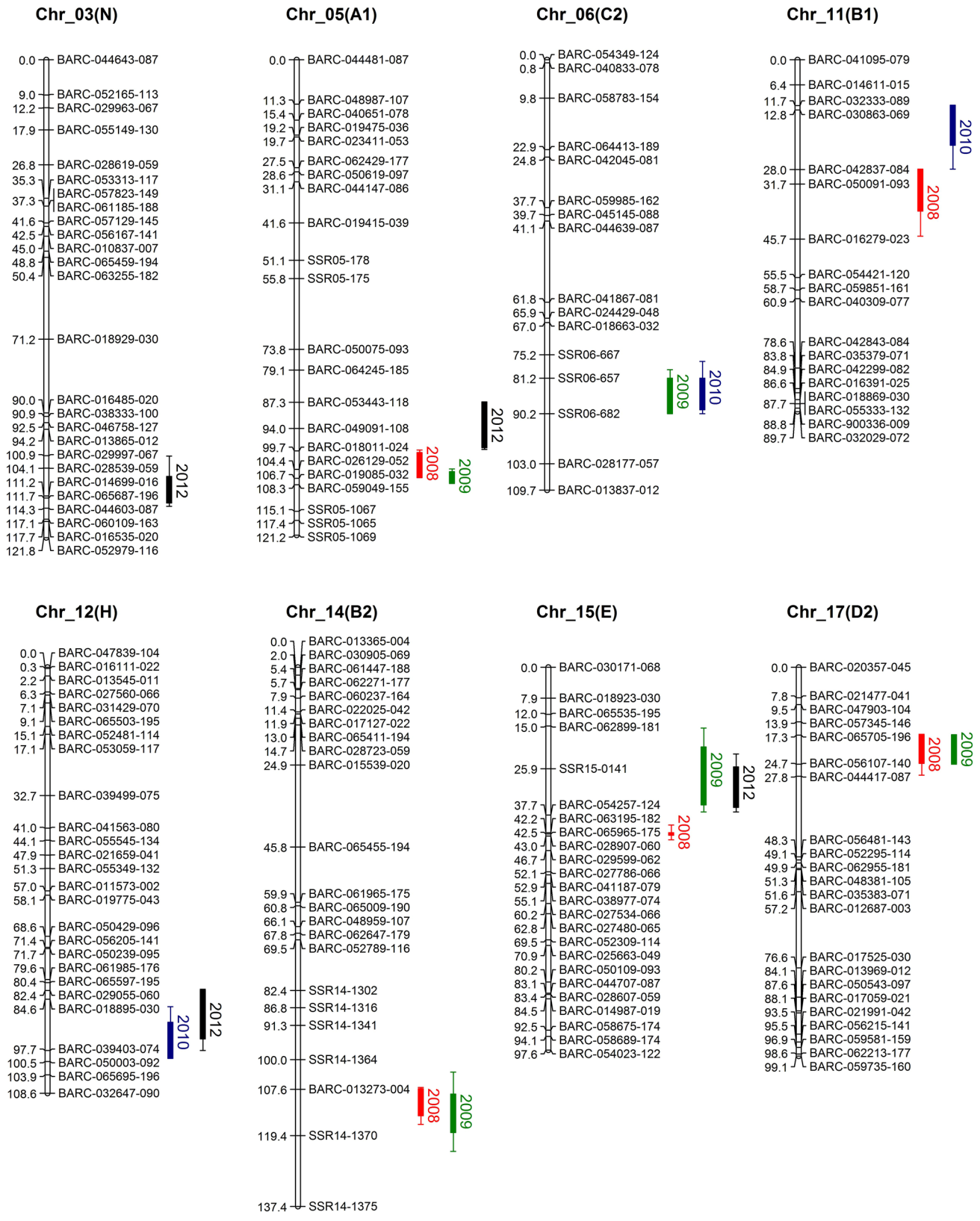

Fig. 2 Locations of QTL conferring ALA concentration detected with CIM in a RIL population derived from a cross between PI483463 × Hutcheson. QTLs are marked with *bars*. The *bar length* represents the support interval of the QTL

Table 4 Estimated additive effect of QTLs for ALA concentration across four environments by mixed model-based composite interval mapping (MCIM) from 188 RILs derived from a PI483463 × Hutcheson at the University of Missouri-Delta Research Center, MO, USA, in 2008 and 2009 and at Gunwi, Korea, in 2010 and 2012

QTL	Chr	Flanking marker	Position (cM)	*A* (%)[a]	h^2 (A) (%)[b]	h^2 (AE) (%)[c]	CIM[d]
qALA3_1	3	BARC-065687-196~BARC-044603-087	112.7	−29**	5.4	0.3	K2
qALA5_3	5	BARC-059049-155~SSR05-1067	111.3	−29**	6.3	0.0	U1, U2
qALA6_1	6	SSR06-657~SSR06-682	84.2	−35**	3.8	0.3	U2, K1
qALA11_2	11	BARC-030863-069~BARC-042837-084	22.8	−26**	5.4	0.4	K1
qALA12_2	12	BARC-039403-074~BARC-050003-092	99.7	−39**	7.9	0.5	K1
qALA13_1	13	BARC-050657-098~BARC-050235-095	78.4	−23**	2.4	0.2	
qALA14_1	14	SSR14-1364~BARC-013273-004	105.0	−30**	4.5	0.6	U1, U2
qALA15_1	15	SSR15-0141~BARC-054257-124	26.9	−36**	5.2	0.2	U2, K2
qALA17_1	17	BARC-065705-196~BARC-056107-140	22.3	−33**	6.0	0.0	U1, U2

** $P < 0.001$

[a] Additive effect: negative values indicate that PI483463 contributed the allele for an increase in the trait value

[b] Phenotypic variance explained by additive QTL

[c] Phenotypic variance explained by additive QTL × environment interaction effect

[d] The numbers U1 and U2, K1 and K2 indicate that the QTL was detected at MO, USA in 2008 and 2009 and Gunwi, Korea in 2010 and 2012 by CIM of WinQTL Cartographer V2.5, respectively

genetic contribution and the identified QTL proportion by the MCIM mapping procedure, which is designated as collectively unmapped QTL (Kim et al. 2014; Xing et al. 2012). The error variation was obtained from the differences between total phenotypic variation, total genetic contribution, variation explained by all detected additive QTL × environment and epistatic QTL × environment. Therefore, the genetic contributions from detected and undetected QTL, additive QTL × environment, epistatic QTL × environment, and error variation are 100 % of the PV.

In Table 6, 61.2 % of the PV for linolenic acid concentration was explained by 46.9 % of additive QTLs, 5.5 % of epistatic QTL pairs, and 8.8 % from the unmapped QTLs. In addition, the additive QTL × year and epistatic QTL × year interaction contributed 0.7–2.5 % of PV to ALA concentration. Therefore, ALA concentration was genetically controlled primarily by a large number of QTLs with additive effects. The additive QTL × year and epistatic QTL × year interaction was very small and negligible.

Discussion

ALA is an essential fatty acid in human and animal diets, and it plays an important role in improved health and disease prevention. Selected wild soybean (*Glycine soja*) accessions contain almost twice the amount of ALA relative to cultivated soybean (USDA, ARS 2007). In the present study, a wild soybean accession, PI483463, contained 16.5 % ALA, while a cultivated soybean cultivar, Hutcheson, contained 9.1 % ALA concentration in the seed oil. Thus, the wild soybean germplasm line could be a useful source for increasing the omega-3 fatty acid ALA concentration in soybean for various soy-food and industrial applications (Lee et al. 2007). It is well known that ALA is synthesized by adding a third double bond to linoleic acid by microsomal omega-3 fatty acid desaturase (FAD3). ALA accumulation in several high (40–68 %) ALA-producing plant species, such as flax (*Linum usitatissimum*), Melissa Moldavica (*Dracocephalum moldavica*), and perilla (*Perilla frutescens*), is associated with much higher omega-3 fatty acid desaturase mRNA levels than that found in soybean (Rao et al. 2008). Therefore, it was suggested that the transcript levels of the FAD3 gene play a key role in ALA accumulation. In soybean, three soybean microsomal omega-3 fatty acid desaturase genes have been identified. In the annotated soybean genome sequence, *GmFAD3A* is represented as Glyma14g37350 on Chr 14, *GmFAD3B* is represented as Glyma02g39230 on Chr 2, and *GmFAD3C* is represented as Glyma18g06950 on Chr 18 (Bilyeu et al. 2011). However, the genetic relationships between the FAD3 genes and high ALA accumulation in wild soybean have not been studied.

In PI483463, nine QTLs significantly associated with ALA concentration were detected by MCIM mapping procedure among the four environments. Five QTLs, *qALA5_3*, *qALA6_1*, *qALA14_1*, *qALA15_1*, and *qALA17_1* located on Chrs 5, 6, 14, 15, and 17, were identified in two environments. Among them, three QTLs, *qALA5_3*, *qALA14_1*, and *qALA17_1* on Chrs 5, 14, and 17 were identified at

Table 5 Estimated epistatic effects of QTLs for ALA concentration from wild soybean in four environments from 188 RILs derived from a PI483463 × Hutcheson at the University of Missouri-Delta Research Center, MO, USA, in 2008 and 2009 and at Gunwi, Korea, in 2010 and 2012

Pair	QTL	Flanking markers	Position (cM)	QTL	Flanking markers	Position (cM)	AA (%)[a]	h^2 (AA) (%)[b]	h^2 (AAE) (%)[c]
1	*qALA13_1*	BARC-050657-098~BARC-050235-095	78.4	*qALA15_1*	SSR15-0141~BARC-054257-K24	26.9	16**	0.7	0.2
2	*qALA4_1*	BARC-038989-074~BARC-044373-086	99.8	*qALA5_1*	BARC-044147-086~BARC-019415-039	32.1	−25**	2.5	0.0
3	*qALA4_2*	BARC-061009-170~BARC-032045-072	139.8	*qALA11_3*	BARC-054421-K20~BARC-059851-161	58.5	25**	2.3	0.5

[a] Epistatic effects, a positive value indicates that the parental two-locus genotypes have a positive effect and that the recombinants have a negative effect

[b] Phenotypic variance explained by epistatic QTL pair

[c] Phenotypic variance explained by epistatic QTL pair × environment interaction effect

** $P < 0.001$

MO, USA in 2008 and 2009. Thus, these three QTLs were most consistently associated with ALA concentration compared to the other putative QTLs with *aALA14_1*, showing the largest PV with 13.8 and 14.3 % in 2008 and 2009, respectively. The other two QTLs, *qALA6_1* and *qALA15_1* were detected in MO, USA in 2009 and Gunwi, Korea in 2010 and 2012. The QTL *qALA6_1* had PVs of 10.0–10.2 % for ALA concentration in 2009 and 2010. This indicated that genetic markers linked to the *qALA6_1* QTL on Chr 6 could be useful for marker-assisted breeding to develop soybeans with high ALA concentration. All of the detected QTLs (Table 3) showed negative additive effects indicating that the alleles from PI483463 contributed to the ALA concentration in soybean seed. The major QTL *qALA15_1* was mapped near a major QTL for ALA concentration that was detected in a wild soybean accession Hidaka 4 (Shibata et al. 2008) and located near the marker Satt384 (19.3 cM on the consensus map). It was located <5 cM from BARC-054257-124 (20.6 cM) which is flanking marker of the QTL *qALA15_1*. Thus, they might be the same QTL controlling ALA concentration.

The QTL *qALA14_1* (47 Mb) was closely linked to *GmFAD3A* (Glyma14g37350), which is located at approximately 47 Mb on Chr 14. The *GmFAD3A* gene was shown to have a higher expression level than the other two desaturase genes (*GmFAD3B* and *GmFAD3C*), with a greater impact on seed linolenic acid (Bilyeu et al. 2003, 2005). The recessive allele associated with the *GmFAD3A* gene caused an approximately 50 % reduction in seed linolenic acid concentrations in A5 and C1640 soybean mutants (Bilyeu et al. 2003; Chappell and Bilyeu 2006). Overexpression of the *GmFAD3A* gene resulted in an approximately two- to fourfold higher ALA concentration in transgenic rice seeds (Liu et al. 2012). Therefore, the high ALA concentration in wild soybean PI483463 might be associated with the high gene expression level of *GmFAD3A* that was closely associated with *qALA14_1*.

Wang et al. (2014) reported QTLs on Chrs 6 (56.5 cM) and 13 (27.9 cM) controlled linolenic acid concentration in cultivated soybeans. In our study, *qALA6_1* and *qALA13*_1 were on Chrs 6 (84.2 cM) and 13 (78.4 cM), respectively. However, the distance of these two QTLs in our study were detected far away from the QTLs reported by Wang et al. (2014). It seems that the QTLs on Chrs 6 and 13 detected in the present study might be new QTLs for ALA concentration in wild soybeans. Diers and Shoemaker (1992) identified marker pA-118, which was associated with linoleic acid concentration at 58.9 cM on Chr 12 and explained 20 % of the phenotypic variation.

The *qALA3_1*, *qALA11_1*, *qALA11_2*, *qALA12_1*, *qALA12_2*, and *qALA17_1*, are the newly discovered QTLs for ALA concentration in soybean. There were reports that there are differences in structural variation and genetic

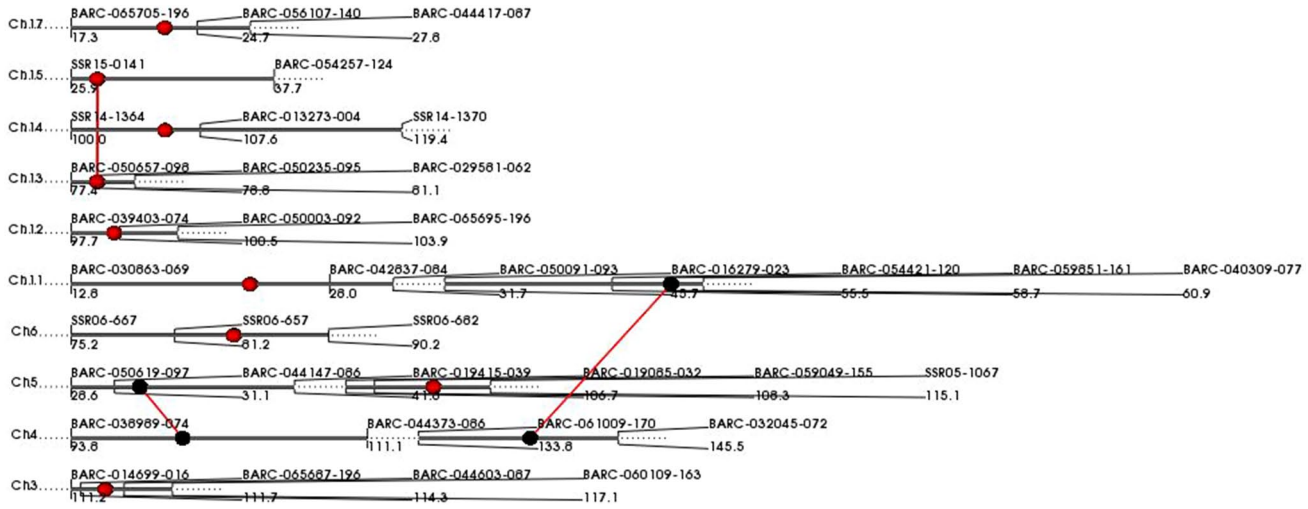

Fig. 3 A representative figure showing epistatic interaction and epistatic QTLs for ALA concentration identified by QTLNetwork V2.0. The *black balls* represent epistatic QTLs without individual effects, while interacting loci are shown by *red lines* with *red balls* (color figure online)

diversity between wild and cultivated soybean (Lam et al. 2010; Kim et al. 2010). The nucleotide and structural variations between the two species might result in finding new QTLs in wild soybean related to accumulation of ALA compared with QTLs in cultivated soybean.

In total, 16 additive effect QTLs and three epistatic QTL pairs were detected that affected ALA. Twelve additive QTLs were detected from CIM and nine additive QTLs and three epistatic QTL pairs were detected from MCIM. Among these QTLs, five additive effect QTLs, *qALA5_3*, *qALA6_1*, *qALA14_1*, *qALA15_1*, and *qALA17_1*, were detected by both the CIM and MCIM mapping procedures and were detected in two of the four environments. The QTL, *qALA6_1*, showed the highest PVs (10.0–10.2 %) compared to the other QTLs detected in two environments. The QTL, *qALA15_1*, was also a component of pair 1 epistatic QTLs (Table 5). Thus these five QTLs, *qALA5_3*, *qALA6_1*, *qALA14_1*, *qALA15_1*, and *qALA17_1*, could be useful in breeding programs by employing marker-assisted selection for high ALA concentration.

Table 6 Contribution of QTL and their interactions to phenotypic variation for ALA concentration (%)

Genetic contribution				Additive QTL × year	Epistatic QTL × year	Error[b]	Total
Additive QTL	Epistatic QTL	Unmapped QTL collectively[a]	Total				
46.9 (9; 76.6)[c]	5.5 (3; 9.0)[c]	8.8 (14.4)[c]	61.2	2.5	0.7	35.6	100

[a] The unmapped QTL portion is calculated from $h^2 - h^2_{(A)} - h^2_{(AA)}$, where h^2 is the heritability estimated from ANOVA and $h^2_{(A)}$ and $h^2_{(AA)}$ are additive and epistatic contributions obtained from the mapping procedure QTLNetwork V2.0, respectively

[b] The error portion was calculated from total phenotypic variation, total genetic contribution, and variation explained by all detected additive QTL × year and epistatic QTL × year

[c] The left numbers in parentheses in the additive QTL and epistatic QTL pair columns are the number of additive QTL and epistatic QTL pairs and the right numbers in parentheses are the percentages of genetic variance explained by the respective QTL, while in unmapped QTL collective column, the number in parentheses is the percentage of genetic variance explained by unmapped QTL collectively. The percentages of the genetic portion explained by additive QTL, epistatic QTL pairs and unmapped QTL collectively are estimated as $\left(h^2_{(A)}/h^2\right) \times 100$, $\left(h^2_{(AA)}/h^2\right) \times 100$ and $\left(h^2 - h^2_{(A)} - h^2_{(AA)}\right)/h^2$, respectively

Additive QTL and epistatic QTL pairs in total explained 52.4 % of the phenotypic variation in ALA concentration by the MCIM procedure (Table 6). The percentage of variation due to additive QTLs and epistatic QTL pairs was 85.6 % for the total genetic variation. This was calculated by dividing the heritability of additive QTL and epistatic QTL pairs (52.4 %) by the heritability (61.2 %) and multiplied by 100. This indicates procedures for the detection of QTLs affecting ALA concentration were reliable.

In conclusion, the detected additive QTLs and epistatic QTL pairs contribute to the genetic contribution of ALA concentration in soybean. It is important to map QTLs associated with ALA in multiple environments from different genetic backgrounds to strengthen the applicability for breeding ALA concentration in soybean. However, this study used a single bi-parental population to detect QTL for ALA concentration. Further confirmation of QTLs in different RIL populations derived from different genetic backgrounds will be necessary for breeding, fine mapping and for gene cloning.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors state that the experiments comply with the current laws of the country in which they were performed.

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