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ARTICLE

Effects of Two Low Phytic Acid Mutations on Seed Quality and Nutritional Traits in Soybean (*Glycine max* L. Merr)

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Reduction of phytic acid in soybean seeds has the potential to improve the nutritional value of soybean meal and lessen phosphorus pollution in large scale animal farming. The objective of this study was to assess the effect of two new low phytic acid (LPA) mutations on seed quality and nutritional traits. Multilocation/season comparative analyses showed that the two mutations did not affect the concentration of crude protein, any of the individual amino acids, crude oil, and individual saturated fatty acids. Among other traits, *Gm-lpa*-TW75-1 had consistently higher sucrose contents (+47.4−86.1%) and lower raffinose contents (−74.2 to −84.3%) than those of wild type (WT) parent Taiwan 75; *Gm-lpa*-ZC-2 had higher total isoflavone contents (3038.8−4305.4 μg/g) than its parent Zhechun # 3 (1583.6−2644.9 μg/g) in all environments. Further tests of homozygous F_3 progenies of the cross *Gm-lpa*-ZC-2 × Wuxing # 4 (WT variety) showed that LPA lines had a mean content of total isoflavone significantly higher than WT lines. This study demonstrated that two LPA mutant genes have no negative effects on seed quality and nutritional traits; they instead have the potential to improve a few other properties. Therefore, these two mutant genes are valuable genetic resources for breeding high quality soybean varieties.

KEYWORDS: *Glycine max* L. Merr.; low phytic acid mutation; seed quality; fatty acids; oligosaccharides; raffinose; stachyose; isoflavones; daidzin; glycitin; genistin

INTRODUCTION

Phytic acid (*myo*-inositol hexa*kis*phosphate, PA) and its salts (phytates) are the major storage form of phosphorus (P) in soybean seeds, like other agriculturally important crops (*1*). Since phytates of important mineral micronutrients, e.g., Zn, Fe, and Ca, cannot be digested by humans and nonruminant livestock, PA is commonly regarded as a major antinutrient in legumes (*1*). In many Asian and other countries, various soybean products are an important part of the daily human diet; therefore, PA may reduce the bioavailability of important mineral micronutrients. In livestock production, most PA-P cannot be utilized by nonruminant livestock and inorganic P (Pi), or microbial phytase (E.C.3.1.3.8) is commonly added in feed to increase available P to animals. Phosphorus leaked from large-scale animal farming to stream and groundwater has become an important source of P pollution. Therefore, low phytic acid (LPA) crops have been developed to improve food/feed nutritional value and ameliorate PA related environment pollution in the past decade (*1*).

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In soybean, LPA mutants have been developed through chemical (*2*, *3*) and physical mutagenesis (*4*), or by using RNAi technology (*5*). Preliminary animal feeding tests showed that the P bioavailability is considerably higher in LPA soybean meals than in normal soybeans (http://www.uky.edu/Ag/AnimalSciences/pubs/soybeanmeal-thegolfstandard.PDF). However, some undesirable agronomic and quality traits, such as lower field emergence rate (*6*, *7*) and greater level of undesirable saturates (palmitate and stearate) in seed oil, were also reported in several LPA soybean lines (*8*). Soybean meals are rich in protein, essential amino acids, insoluble fibers, edible oil, omega-3 fatty acid, fat-soluble vitamins, oligosaccharides, and isoflavones (*9*), and these ingredients can have intrinsic interactions (*10*). Therefore, reduction of phytic acid might have unforeseen consequences on other seed components.

We recently developed two LPA soybean mutant lines, *Gm-lpa*-TW-1 and *Gm-lpa*-ZC-2 (*4*). *Gm-lpa*-TW-1 carries a null mutation of the D-*myo*-inositol 3-phosphate synthase gene 1 (*MIPS1*), but its field emergence rate is less affected than LR33, another LPA line of which *MIPS1* is mutated (*4*). *Gm-lpa*-ZC-2, carrying an LPA mutation different from that

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of other mutants, has yields and agronomic traits almost identical to those of its wild type parent (*4*). Therefore, these two mutations are unique in the sense of their agronomic performance. In this study, effects of the two LPA mutations on seed quality and nutritional traits were investigated with the aim to objectively assess their value for soybean improvement and to detect any possible interaction between phytic acid and other traits.

MATERIALS AND METHODS

Plant Material and Seed Production. Two LPA soybean mutant lines, *Gm-lpa*-TW75-1 (hereafter TW-M) and *Gm-lpa*-ZC-2 (hereafter ZC-M), and their corresponding wild type (WT) parental varieties, Taiwan 75 and Zhechun # 3, were used in this experiment. Both TW-M and ZC-M were developed using gamma irradiation (*4*). Taiwan 75 is a vegetable soybean variety widely grown in Zhejiang Province, and Zhechun # 3 is a spring season soybean variety with a high protein content widely grown in Zhejiang and other provinces of southern China. ZC-M was crossed with a conventional WT soybean variety Wuxing # 4, the F_1 and F_2 plants were grown individually, and F_3 seeds were harvested on individual F_2 plants.

Seed samples used for comparative analysis were always harvested from plants grown in neighboring plots in the same field. Seed production locations and seasons included 2004 spring and autumn seasons in Hangzhou, Zhejiang in the fields of the Experimental Farm of Zhejiang Academy of Agricultural Sciences, and the 2005/6 winter/spring season in Lingshui, Hainan in the Winter Breeding Nursery of Zhejiang University.

Seed P Phenotyping. Seed P phenotype was determined through a qualitative colorimetric assay of inorganic P (Pi) using freshly prepared Chen's reagent (*11*) according to Wilcox et al. (*2*), with slight modifications (*4*). Development of a blue color implies a high Pi level, typical for LPA mutants, and colorlessness typifies a normal Pi level as WT parent varieties (*ref4*). Seeds with a high Pi level are hereafter referred to as LPA (mutant) seeds and those with a normal Pi level as WT seeds. For the F_3 seeds of the ZC-M × Wuxing #4 cross, six seeds from each F_2 plant were analyzed for the Pi level. If all six tested seeds showed a high or normal Pi level, then the corresponding F_3 seeds were recorded as homozygous LPA or WT lines and used for further analysis. As background information, the PA content data in **Table 1** was adopted from Yuan et al. (*4*) and was measured using anion-exchange high performance liquid chromatography (HPLC) (*12*).

Determination of Crude Protein and Oil Content. The content of crude protein and oil was determined by near-infrared spectrometry using intact soybean seeds. Twenty grams of soybean seeds were loaded each time and scanned 10 times for each sample on Infratec 1241 (FOSS Analytical AB Hoganas, Sweden). The equipment came with ready-to-use calibrations for protein, moisture, starch, and oil, and calibrations for local use were selected. The measurement was repeated 3 times; data of protein and oil content were presented on a dry weight basis (0% moisture) for comparison.

Determination of Amino Acids, Fatty Acids, Isoflavones, and Oligosacchrides. *Sample Preparation*. Mature seeds were ground into soybean flour in a Cyclone Mill (UDY Corporation, Fort Collins, CO, USA) passing through a 60-mesh screen. The flour was freeze-dried for 48 h and stored at −18 °C until subject to analysis.

Amino Acids. The content of various amino acids was determined using an Automatic Amino Acid Analyzer (HITACHI 835-50) according to the Chinese national standard protocol for amino acid determination (GB/T 5009.124-2003). Briefly, 1g of soybean flour was hydrolyzed for 22 h in a vacuum glass tube, using 15 mL of 6 mol/L hydrochloric acid with 3−4 drops of phenol at 110 °C. The hydrolysate was filtrated and transferred to a 50 mL volumetric flask and adjusted to full volume with ddH_2O. One milliliter of hydrolysate was transferred to a 5 mL volumetric flask and dried in vacuum at 50 °C. The residue was dissolved in 5 mL of sodium citrate buffer (pH 2.2) and determined by an automatic amino acid analyzer, together with standard amino acid samples.

Table 1. Content of Crude Protein and Crude Oil in Seeds of Mutants and Their Wild Type Parents from Different Growth Seasons/Locations (mg/g)[a]

materials	phytic acid	inorganic P	crude protein	crude oil
Hangzhou, Spring Season, 2004				
Zhechun No3	13.2 ± 0.0**	0.5 ± 0.0**	463.1 ± 0.1	163.6 ± 0.2
Gm-lpa-ZC-2	9.7 ± 0.1	1.4 ± 0.0	472.1 ± 0.2	167.4 ± 0.2
Taiwan 75	14.0 ± 0.4**	0.5 ± 0.0**	416.9 ± 0.2	200.6 ± 0.3
Gm-lpa-TW75-1	6.4 ± 0.1	3.7 ± 0.1	410.5 ± 0.4	200.9 ± 0.4
Hangzhou, Autumn Season, 2004				
Zhechun No3	11.2 ± 0.6**	0.6 ± 0.0**	432.8 ± 0.3	186.7 ± 0.2
Gm-lpa-ZC-2	5.6 ± 0.1	1.5 ± 0.0	439.9 ± 0.5	192.8 ± 0.1
Taiwan 75	12.2 ± 0.2**	0.6 ± 0.0**	382.1 ± 0.3	226.8 ± 0.2
Gm-lpa-TW75-1	3.7 ± 0.1	4.0 ± 0.2	382.5 ± 0.2	225.9 ± 0.3
Lingshui, Winter/Spring Season, 2004/2005				
Zhechun No3	14.0 ± 0.5**	0.7 ± 0.0**	430.9 ± 0.3	186.4 ± 0.3
Gm-lpa-ZC-2	7.2 ± 0.1	2.3 ± 0.0	432.1 ± 0.2	176.1 ± 0.5
Gm-lpa-TW75-1	3.0 ± 0.1	5.3 ± 0.1	382.8 ± 0.2	219.6 ± 0.3

[a] ** indicates that the phytic acid or Pi level of the parental variety was significantly different from that of its mutant, grown at the same location ($P = 0.01$).

Fatty Acids. The content of various fatty acids was determined using gas chromatography (GC) according to Spencer et al. (*13*) with slight modifications. Briefly, crude oil was extracted from about 1 g of soybean flour with 20 mL of diethyl ether (12 h) at room temperature (~20 °C). Fatty acids were methylesterificated using NaOH in methanol at 60 °C for 5 min, and the resultant fatty acid methyl esters were analyzed on GC (Agilent 6890 series, USA) with a capillary column (15 m, ϕ 0.25 mm). The temperature of the capillary column was set at 150 °C for 4 min and increased at 10 °C per min to 270 °C. The flow rate for air, hydrogen, and helium was set to 400, 30, and 0.7 mL/min, respectively. Aliquots of 5 μL were injected into the capillary column.

Isoflavones. The concentration of various isoflavones was determined by HPLC (Aglient 110 series) according to Charron et al. (*14*) with slight modifications. Briefly, 5 mL of methanol/0.1 M HCL (3:1 v/v) solution was added into a tube with 0.5 g of soybean flour and mixed by shaking for 2 h at 200 rpm. A 2 mL mixture was transferred to a new tube and centrifuged at 10,000 rpm at 4 °C for 15 min. The clear aliquot was filtered through a 0.45-μm PTFE filter. The analysis was performed under the following instrumental conditions: mobile phases were solvent A (0.1% v/v trifluoro acetic acid/ddH_2O) and solvent B (0.1% v/v trifluoro acetic acid/acetonitrile) with a solvent system (% solvent A/% solvent B) for 14 min (71/29), 18 min (70/30), 24 min (58/42), and 34 min (100/0) with a flow rate of 1 mL/min and an injection volume of 5 μL. Twelve isoflavone standards were used and purchased from Sigma USA (daidzin, glycitin, genistin, M-daidzin, M-glycitin, A-daidzin, A-glycitin, M-geinistin, daidzein, glycitein, A-genistin, and genistein).

Oligosaccharides. The content of various oligosaccharides was determined by HPLC according to Xia et al. (*15*), with slight modifications. Briefly, 10 mL of 80% ethanol was added into a 50 mL flask with 1 g of defatted flour and mixed by shaking at 60 °C for 2 h. The mixture was transferred to a 50 mL tube and centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was transferred to a new 50 mL tube and the precipitate was re-extracted twice with 10 mL of 80% ethanol. All of the supernatant was combined, and the protein was precipitated by the addition of 1 mL of saturated lead acetate trihydrate and

adjusting pH to 4−5; The redundant Pb^{2+} in the supernatant was removed by adding 0.6 mL of 0.5 N oxalic acid dihydrate. The mixture was then centrifuged at 10,000 rpm at 4 °C for 10 min, and the supernatant was transferred to one clean tube, and the pH was adjusted to 7.0 with 1 N NaOH. The ethanol was vaporized at 85 °C for about 6 h, and its volume was adjusted to 5 mL. After passing through a 0.2 μm filter, aliquots were fractionated on HPLC (Aglient 110 series), which had already been equilibrated with 65% acetonitrile at the rate of 1 mL/min. External standards of oligosaccharides, i.e., sucrose, raffinose, and stachyose (Sigma, USA), were analyzed before and after every two samples.

Three independent flour samples of each soybean line produced at a given location/season were used for the determination of the above-mentioned traits; three measurements were made for each flour sample. The mean value was calculated from three flour samples of each treatment.

Statistical Analysis. Multiple comparison analysis was performed using the Statistical Analysis System (SAS 8.0 Institute, Inc., Cary, NC, USA). Data was expressed as the mean with standard deviation (SD) and compared by one-way analysis of variance (ANOVA), followed by the Duncan test.

RESULTS

Seed Composition of LPA Mutants and WT Parents. Although there were substantial effects of growing locations and seasons, the concentrations of both PA and Pi content of the two LPA mutant lines were significantly different from their corresponding WT parental varieties (**Table 1**). These results suggested that the two LPA mutations resulted in reduced PA and increased Pi concentrations in all tested environments.

Crude Protein and Amino Acids. The two mutant lines had crude protein content very similar to that of their respective WT parental lines grown in the same location/season, although significant environmental (season and location) and genotypic effects were also observed (**Table 1**). In addition, no significant concentration differences were detected between LPA mutants and their respective WT parents for each of the individual amino acids analyzed (data not shown). These results implied that the two LPA mutations had no significant effect on seed protein and individual amino acids.

Crude Oil and Fatty Acids. Similar to protein content, no significant differences of crude oil concentration were observed between the two LPA lines and their respective WT parental lines, although significant environmental and genotypic effects were also observed (**Table 1**).

Five major fatty acids, palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3), were detected in the crude oil of soybean seeds of both LPA mutants and WT parents; other fatty acids, such as arachidic (C20:0) and behenic (C22:0) were minor and not included for further analysis. The concentration of the major five fatty acids varied between the two parental varieties and among seeds produced in different seasons/locations, implying the existence of both genotypic and environmental effects (**Table 2**). No significant differences were detected for the two saturated fatty acids and total unsaturated fatty acids between LPA lines and their respective WT parental varieties in all locations/seasons; however, significant differences were observed for the unsaturated fatty acids in certain locations/seasons (**Table 2**).

The oleic acid concentration of ZC-M was lower than Zhechun # 3 in all the tested environments, with a reduction of from 3.8% to 15.7% (significant in both seasons in Hangzhou, **Table 2**). Significant differences of oleic acid concentration between TW-M and its parent were only observed in the 2004 spring season in Hangzhou, where the mutant was 30.1% higher than the WT (**Table 2**). Significant differences of linolenic acid concentration were also observed between the two sets of mutant and parent, but the trend of differences appeared to be almost in the direction opposite to that of oleic acid (**Table 2**). For example, the contents of oleic acid of Zhechun # 3 were significantly higher, and those of linolenic acids were significantly lower than those of ZC-M in both seasons of 2004 in Hangzhou (**Table 2**). Significant differences of linoleic acid concentrations were only observed in seeds produced in the spring season of 2004 in Hangzhou for both mutant lines with an 5.7% increase for ZC-M and a 23% decrease for TW-M compared with their respective parental lines (**Table 2**).

Oligosaccharides. The mutant ZC-M had oligosaccharide concentrations similar those of to its parent Zhechun #3 in

Table 2. Average Fatty Acid Composition Rations in Crude Oil of Mutants and Their Wild Type Parents from Different Growth Seasons/Locations (mg/g)[a]

	saturated fatty acid			unsaturated fatty acid			
materials	palmitic acid (C16:0)	stearic acid (C18:0)	subtotal	oleic acid (C18:1)	linoleic acid (C18:2)	linolenic acid (C18:3)	subtotal
			Hangzhou, Spring Season, 2004				
Zhechun No3	132.3 ± 0.5	30.1 ± 0.1	162.4 ± 0.2	332.1 ± 0.4*	456.6 ± 0.3*	48.9 ± 0.4*	837.6 ± 0.4
Gm-lpa-ZC-2	139.6 ± 0.5	30.8 ± 0.2	170.4 ± 0.2	287.0 ± 0.6	482.8 ± 0.4	54.5 ± 0.7	824.3 ± 0.4
Taiwan 75	103.5 ± 0.4	23.1 ± 0.2	126.6 ± 0.3	400.5 ± 1.0*	411.8 ± 0.6*	55.4 ± 0.3*	873.4 ± 0.5
Gm-lpa-TW75-1	100.9 ± 0.2	24.3 ± 0.2	125.2 ± 0.1	522.7 ± 0.4	316.9 ± 0.5	34.4 ± 0.3	874.8 ± 0.3
			Hangzhou, Autumn Season, 2004				
Zhechun No3	130.6 ± 0.5	38.3 ± 0.1	168.9 ± 0.4	216.2 ± 1.0*	532.7 ± 0.5	79.2 ± 0.5*	831.1 ± 0.6
Gm-lpa-ZC-2	125.1 ± 1.0	35.6 ± 0.2	160.7 ± 0.4	188.9 ± 2.3	542.2 ± 3.7	105.3 ± 1.7	839.3 ± 1.6
Taiwan 75	120.7 ± 0.6	24.8 ± 0.1	145.5 ± 0.4	304.4 ± 0.4	498.5 ± 0.5	51.2 ± 1.3*	854.5 ± 0.9
Gm-lpa-TW75-1	120.2 ± 0.6	24.8 ± 0.2	145 ± 0.5	290.4 ± 1.2	501.5 ± 2.5	75.8 ± 1.4	855 ± 1.9
			Lingshui, Winter/Spring Season, 2004/2005				
Zhechun No3	128.0 ± 0.6	32.9 ± 0.2	160.9 ± 0.4	203.9 ± 0.6	546.9 ± 0.5	84.7 ± 0.9	839.1 ± 0.8
Gm-lpa-ZC-2	137.8 ± 0.5	34.5 ± 0.1	172.3 ± 0.3	196.5 ± 0.5	543.8 ± 0.7	85.0 ± 0.7	827.7 ± 0.8
Gm-lpa-TW75-1	113.6 ± 0.7	26.9 ± 0.2	140.5 ± 0.6	348.2 ± 1.1	454.6 ± 0.7	75.3 ± 0.6	859.5 ± 1.0

[a] *Fatty acid level of the parental variety was significantly different from that of its mutant, grown at the same location ($P = 0.05$).

all environments except 2004 autumn in Hangzhou, where the mutant had significantly increased raffinose concentration and reduced stachyose concentration. However, the sum of the two raffinosaccharides remained unchanged (**Table 3**).

Compared to its parent Taiwan 75, TW-M had significantly greater concentrations of sucrose (+47.4−86.1%) and reduced concentrations of both raffinosaccharides (**Table 3**).The raffinose contents of the mutant were only about 20% that of the parent, while stachyose contents were almost diminished in the mutant (**Table 3**). Although no seeds were produced for Taiwan 75 in Hainan, the increase of sucrose and reduction of raffinose and stachyose might even be more significant than in the other two locations since the sucrose of TW-M was much higher, and the raffinose was much lower in other two environments (**Table 3**).

Table 3. Content of Oligosaccharides in Seeds of Mutants and Their Wide Type Parents from Different Growth Seasons/Locations (mg/g)[a]

materials	sucrose	raffinose	stachyose
	Hangzhou, Spring Season, 2004		
Zhechun No3	53.9 ± 1.1	6.4 ± 0.1	10.3 ± 0.0
Gm-lpa-ZC-2	43.8 ± 1.5	6.0 ± 0.3	9.9 ± 0.4
Taiwan 75	50.2 ± 0.5*	11.0 ± 0.6**	10.3 ± 0.5
Gm-lpa-TW75-1	74.0 ± 1.7	2.1 ± 0.1	not detected
	Hangzhou, Autumn Season, 2004		
Zhechun No3	62.7 ± 3.1	7.3 ± 0.2*	13.7 ± 0.3*
Gm-lpa-ZC-2	57.4 ± 0.3	4.7 ± 0.3	18.2 ± 0.6
Taiwan 75	44.7 ± 0.6**	10.6 ± 0.3**	11.3 ± 1.2**
Gm-lpa-TW75-1	83.3 ± 0.7	2.7 ± 0.1	0.8 ± 0.1
	Lingshui, Winter/Spring Season, 2004/2005		
Zhechun No3	60.3 ± 1.0	7.3 ± 0.2	16.0 ± 0.3
Gm-lpa-ZC-2	67.7 ± 0.9	7.4 ± 0.4	15.7 ± 0.2
Gm-lpa-TW75-1	112.2 ± 0.1	1.8 ± 0.2	not detected

[a] * and ** indicate that the oligosacchrides level of the parental variety was significantly different from that of its mutant, grown at the same location at $P = 0.05$ and 0.01, respectively.

Isoflavones. Various isoflavones were detected in soybean seeds of both LPA mutants and their WT parents. For simplicity, glucosides were presented as the sum of all three forms, e.g., daidzin representing daidzin, malonyl daidzin, and acetyl daidzin (**Table 4**).

Total Isoflavone. The concentrations of total isoflavone of both TW-M and ZC-M differed significantly from their respective WT parents (**Table 4**). ZC-M had consistently higher total isoflavone concentration (+28.8−178.9%) than Zhechun # 3 in all of the environments. TW-M had a significantly higher concentration in the spring but a lower one in the autumn season in Hangzhou (**Table 4**).

Glucosides. The concentrations of total glucoside were increased in ZC-M across all environments compared to those of its parent, with the least increase in Lingshui (37.5, 1.6, and 36.0% for daidzin, glycitin, and genistin, respectively) and the greatest in Hangzhou in autumn (233.0, 44.8, and 197.8%, for daidzin, glycitin, and genistin, respectively) (**Table 4**). Among the glucosides, the most significant change was observed for daidzin.

Aglycones. The concentrations of various aglycones were quite low (not detectable on some occasions) compared with those of glucosides; although significant differences were observed between the two mutants and their respective parents, there was no consistent trend of either increase or decrease of any aglycones across the three environments (**Table 4**).

Isoflavone Concentration of Progenies. To determine whether the consistently higher isoflavone concentrations of ZC-M over its wild type Zhechun # 3 was due to the pleiotropic effect of the LPA mutation, 13 homozygous LPA and 12 WT $F_{2:3}$ lines of the cross *Gm-lpa*-ZC-2 × Wuxing #4 were subjected to an analysis of isoflavone concentrations.

LPA lines had a mean total isoflavone concentration of 2831.9 μg/g, which is significantly higher than the average of 1444.9 μg/g of the WTs (**Table 5**). Overall, LPA lines had a 96.7% and 73.9% increase of glucosides and algycones, respectively, compared to the WT lines. Similar to ZC-M versus Zhechun # 3, the greatest difference was observed for daidzin and the least for glycitin (**Table 5**). However, there

Table 4. Content of Total and Individual Isoflavones in LPA Soybean Seeds and Their Wild Type Parents (μg/g) [a]

	glucoside (including malonyl and acetyl glucosides)				aglycon				
materials	daidzin	glycitin	genistin	subtotal	daidzein	glycitein	genistein	subtotal	total
				Hangzhou, Spring Season, 2004					
Zhechun No3	707.9 ± 6.1*	321.8 ± 10.0*	684.6 ± 1.1*	1713.8 ± 2.7**	11.5 ± 0.8*	20.7 ± 0.2*	13.5 ± 6.5*	45.8 ± 7.6*	1764.6 ± 2.9*
Gm-lpa-ZC-2	1447.8 ± 6.1	367.4 ± 5.0	1158.1 ± 10.0	2973.4 ± 2.1	19.6 ± 1.2	36.1 ± 0.9	9.7 ± 0.3	65.4 ± 2.4	3038.8 ± 2.3
Taiwan 75	57.9 ± 1.8	16.8 ± 1.7*	230.9 ± 5.1*	305.7 ± 8.6*	NT	NT	NT	NT	305.7 ± 8.6*
Gm-lpa-TW75-1	59.8 ± 3.9	22.8 ± 2.8	120.3 ± 6.7	202.9 ± 3.9	NT	NT	NT	NT	202.9 ± 3.4
				Hangzhou, Autumn Season, 2004					
Zhechun No3	568.3 ± 5.8**	320.3 ± 3.4*	619.5 ± 4.7*	1508.0 ± 1.4**	18.6 ± 0.4*	57.1 ± 0.6*	NT	75.6 ± 0.9*	1583.6 ± 1.5**
Gm-lpa-ZC-2	1892.3 ± 6.4	463.9 ± 4.1	1844.9 ± 7.8	4201.1 ± 8.2	14.6 ± 0.7	89.7 ± 3.6	NT	104.3 ± 4.4	4305.4 ± 8.5
Taiwan 75	274.6 ± 5.3**	135.9 ± 5.0*	418.0 ± 2.7**	828.6 ± 8.2**	NT	31.8 ± 0.2**	NT	31.8 ± 0.2**	860.4 ± 3.1**
Gm-lpa-TW75-1	716.4 ± 1.2	97.1 ± 6.9	949.9 ± 3.3	1763.4 ± 2.5	20.3 ± 1.7	94.2 ± 7.4	38.8 ± 3.1	152.3 ± 2.2	1916.7 ± 3.7
				Lingshui, Winter/Spring Season, 2004/2005					
Zhechun No3	1040.6 ± 5.4*	474.5 ± 3.1	1032.1 ± 8.1*	2547.2 ± 8.3*	10.1 ± 0.3*	87.7 ± 0.7*	NT	97.8 ± 0.9	2644.9 ± 7.3*
Gm-lpa-ZC-2	1430.9 ± 7.8	482.3 ± 6.5	1403.3 ± 8.0	3316.6 ± 5.3	14.3 ± 0.8	76.7 ± 0.9	NT	90.9 ± 1.6	3407.6 ± 9.2
Gm-lpa-TW75-1	1021.9 ± 6.5	236.7 ± 5.3	1097.9 ± 8.6	2356.6 ± 6.2	15.5 ± 0.6	61.0 ± 1.3	NT	76.6 ± 1.8	2433.1 ± 7.2

[a] **and * indicate that the isofalvones level of the parental variety was significantly different from that of its mutant, grown at the same location at $P = 0.01$ and 0.05, respectively. NT: not detected.

Table 5. Total and Individual Isoflavone Contents in Seeds of LPA/Non-LPA Lines Derived from the Mutants (μg/g)

	glucosides (including malonyl and acetyl glucosides)				algycones				
$F_{2:3}$ lines	daidzin	glycitin	genistin	subtotal	daidzein	glycitein	genistein	subtotal	total
				Homozygous Low Phytic Acid Lines					
77027−1	595.3 ± 2.0	319.8 ± 2.9	581.4 ± 3.9	1496.5 ± 2.7	17.4 ± 3.0	17.0 ± 1.9	11.6 ± 0.4	46.1 ± 5.3	1542.6 ± 4.3
77031−1	609.8 ± 3.2	315.5 ± 4.7	1341.9 ± 4.9	2197.8 ± 7.2	NT[a]	21.8 ± 1.5	NT	21.8 ± 1.5	2219.6 ± 4.3
77031−2	916.3 ± 6.7	447.0 ± 4.6	1561.9 ± 6.4	2478.2 ± 7.7	23.4 ± 2.4	33.5 ± 0.9	28.1 ± 0.5	85.1 ± 3.8	2563.3 ± 4.9
77031−3	1677.8 ± 6.0	497.2 ± 3.3	1679.4 ± 3.6	3854.3 ± 6.3	51.2 ± 2.1	27.4 ± 1.8	40.6 ± 0.2	119.2 ± 4.0	3973.4 ± 6.9
77031−7	1408.8 ± 7.0	528.6 ± 8.3	1290.4 ± 4.9	3227.8 ± 6.8	30.8 ± 1.7	41.9 ± 2.8	19.1 ± 0.8	91.8 ± 3.0	3319.6 ± 5.6
77032−1	1056.4 ± 4.9	654.7 ± 6.6	1451.9 ± 0.8	3162.9 ± 2.3	33.5 ± 0.5	32.0 ± 0.8	26.9 ± 0.4	92.5 ± 1.7	3255.4 ± 4.0
77032−2	1124.3 ± 4.5	616.9 ± 4.1	1438.8 ± 2.0	3181.1 ± 5.9	31.7 ± 0.4	30.8 ± 0.5	NT	62.5 ± 0.8	3243.7 ± 6.7
77032−4	1121.8 ± 3.4	530.4 ± 3.1	1393.2 ± 3.2	3045.4 ± 7.2	20.3 ± 1.0	43.1 ± 0.9	18.8 ± 0.2	82.1 ± 2.1	3123.4 ± 1.2
77032−6	1099.3 ± 1.4	558.1 ± 3.6	1651.7 ± 3.0	3198.5 ± 8.0	99.4 ± 1.3	39.5 ± 0.1	38.3 ± 0.5	177.2 ± 1.9	3375.6 ± 8.5
77033−2	626.5 ± 2.7	399.2 ± 4.3	961.4 ± 3.7	1987.1 ± 6.0	20.3 ± 0.6	26.3 ± 0.1	17.9 ± 0.5	64.7 ± 1.2	2051.9 ± 1.2
77033−3	1397.5 ± 4.2	594.5 ± 2.4	1682.9 ± 4.8	3674.9 ± 5.7	34.1 ± 1.8	52.1 ± 0.4	23.8 ± 1.1	109.9 ± 3.3	3784.7 ± 1.9
77034−1	299.3 ± 3.9	374.5 ± 4.7	589.3 ± 3.6	1263.1 ± 2.2	3.7 ± 0.5	28.7 ± 0.9	10.3 ± 0.4	42.7 ± 1.7	1305.7 ± 3.9
77034−4	1122.1 ± 1.3	497.1 ± 1.0	1355.2 ± 0.6	2974.3 ± 2.1	24.8 ± 0.7	38.2 ± 1.1	17.9 ± 0.7	80.9 ± 2.1	3055.3 ± 1.9
average	1004.3 ± 386.9	487.2 ± 109.8	1306.1 ± 373.5	2749.4 ± 803.2	30.1 ± 24.7	33.3 ± 9.6	17.7 ± 11.2	82.8 ± 39.3	2831.9 ± 830.4
				Homozygous Wild Type Lines					
77037−1	210.0 ± 3.0	202.9 ± 2.0	457.7 ± 1.9	870.6 ± 2.4	42.5 ± 0.3	41.3 ± 0.6	63.0 ± 1.2	146.9 ± 2.3	1017.5 ± 2.0
77037−3	266.3 ± 2.0	225.8 ± 2.6	490.4 ± 1.1	982.7 ± 2.6	6.5 ± 0.8	13.1 ± 0.2	5.3 ± 0.1	24.8 ± 1.1	1007.5 ± 2.3
77040−1	413.0 ± 2.1	489.0 ± 2.1	619.8 ± 2.3	1521.8 ± 3.0	5.6 ± 0.7	26.4 ± 0.5	6.5 ± 0.1	38.5 ± 1.2	1560.4 ± 2.4
77040−2	208.2 ± 2.3	420.8 ± 2.8	184.7 ± 2.1	813.6 ± 2.4	15.7 ± 0.7	17.1 ± 0.2	14.9 ± 0.4	47.6 ± 1.3	861.2 ± 2.5
77040−3	667.6 ± 0.9	427.6 ± 1.3	771.8 ± 0.8	1867.0 ± 3.	15.4 ± 0.5	26.3 ± 0.2	9.8 ± 0.3	51.5 ± 0.9	1918.4 ± 3.1
77045−1	274.9 ± 2.5	324.8 ± 2.7	612.2 ± 1.5	1211.9 ± 2.7	11.8 ± 0.3	19.9 ± 0.7	10.1 ± 0.9	41.6 ± 1.9	1253.6 ± 3.0
77045−2	195.8 ± 0.5	407.9 ± 1.7	559.3 ± 0.7	1163.0 ± 2.9	NT	15.1 ± 0.5	NT	15.1 ± 0.5	1178.2 ± 3.5
77045−3	257.9 ± 1.9	355.7 ± 2.3	550.4 ± 1.3	1164.0 ± 3.6	11.2 ± 0.2	16.7 ± 0.5	8.2 ± 0.6	36.0 ± 1.3	1200.2 ± 3.3
77045−11	267.7 ± 2.6	402.7 ± 1.3	574.4 ± 1.9	1294.1 ± 3.8	9.0 ± 0.6	12.5 ± 0.5	9.1 ± 0.2	30.6 ± 1.3	1324.7 ± 2.6
78003−2	488.5 ± 2.1	519.8 ± 2.4	839.4 ± 2.1	1847.8 ± 3.2	22.8 ± 0.9	NT	22.8 ± 0.9	22.8 ± 0.9	1870.6 ± 4.1
78003−3	563.9 ± 1.0	461.4 ± 1.2	728.3 ± 1.9	1753.7 ± 4.1	22.3 ± 0.4	28.4 ± 0.5	5.3 ± 0.2	56.1 ± 1.1	1809.6 ± 2.3
78003−4	732.1 ± 1.6	535.9 ± 0.9	1009.9 ± 1.9	2277.8 ± 4.4	18.1 ± 0.7	29.5 ± 0.1	11.9 ± 0.2	59.6 ± 1.0	2337.5 ± 3.2
average	378.8 ± 181.9	397.9 ± 101.1	616.5 ± 199.6	1397.3 ± 436.4	15.1 ± 10.5	20.5 ± 10.2	13.9 ± 15.8	47.6 ± 32.7	1444.9 ± 432.6

[a] NT: not detected.

were also a few LPA lines having isoflavone concentrations comparable to those of the WT lines (**Table 5**); for example, the total isoflavone concentration of 4 LPA lines were lower than that in the highest WT line (2337.5 μg/g) (**Table 5**).

DISCUSSION

Reduction of phytic acid has potential benefits both for animal nutrition and environment protection, but it should not be achieved at the cost of other important traits. Therefore, any potential negative effects of newly identified or induced low phytic acid mutant genes should be evaluated before they are widely used in breeding programs to develop commercial varieties. Two types of LPA soybean were previously reported: LR33 with a 50% reduction in phytate resulting from a G/T base change in the exon region of the MIPS1 gene (*3*) and M153 with a reduction ≥75% of seed phytate and conditioned by two nonallelic mutant alleles *pha1* and *pha2* (*2*, *16*). The two LPA lines used in this study contain mutant genes different from LR33 and M153. TW-M carries with a mutant allele of the MIPS1 gene (a 2 bp deletion in the third exon) and ZC-M has a recessive mutation located in linkage group (LG) B2 (*4*). Therefore, it was expected they might have effects on seed compositional components different from those reported for either LR33 or M153.

Protein is one of the major components of soybean seeds. It has been well demonstrated that phytate is deposited in protein storage vacuoles (*1*). Earlier studies indicated that protein content is highly correlated with phytic acid concentration in soybean, maize, and winter wheat (*17*), although no such relationship was observed in rice (*18*). Our results showed that both LPA mutations had no effect on protein concentration, which is consistent with our observation on LPA rice (*19*).

Oil is another major component in soybean seeds, and its quality requirement is determined by its end-use. High saturated oil is preferred in industrial use, e.g., for deep frying and production of solid or semisolid fats (*20*), while oil with high unsaturated fatty acid is used as edible oil or for food processing. LPA mutant line CX1834 and its derived LPA lines had significantly higher palmitate and stearate concentrations than those of the WT lines, which is regarded as a negative effect of the CX1834 *lpa* mutation because its oil is not suitable for either edible use or food processing (*8*). In this study, the total saturated and unsaturated fatty acid concentrations of the two mutant lines were not significantly different from their corresponding parental varieties (**Table 2**). A lower concentration of linolenic acid is preferred in the food processing industry since linolenic acid is known to be a major cause of the oxidative instability of cooking oil (*21*). Significant differences of linolenic acid concentrations were only observed in one of the three environments between ZC-M and Zhechun # 3 (Hangzhou, autumn season), and the differences between TW-M and Taiwan 75 were not consistent over two seasons, increased in one location and decreased in the other (**Table 2**). Therefore, it can be concluded that the two LPA mutations have no significant effect on the properties of soybean oil.

Carbohydrates may play a role in the acquisition of desiccation tolerance and storability of soybean seeds, and they are also important quality traits of soybean meals since raffinose

and stachyose (raffinosaccharides) are not digested by higher animals including humans and can cause digestive disturbances and depress the growth of early weaned pigs (*9*).

The metabolism of phytic acid and carbohydrates is interlinked. The first step in the synthesis of phytic acid is the conversion of D-glucose 6-phosphate to myo-inositol-3-phosphate [Ins(3)P1], catalyzed by MIPS, followed by phosphorylation steps (*1*)). Raffinose is synthesized by attaching galactose to sucrose, involving *myo*-inositol as a carrier of galactose, activated as galactinol, and stachyose is a further extension of raffinose (*22*). It has also been proven that the accumulation of raffinosaccharides was controlled by the levels of the initial substrates, i.e., sucrose and *myo*-inositol (*23*). Therefore, *myo*-inositol, raffinose, and galactose are metabolites clearly linked to the biogenetic pathways leading to phytic acid (*24*). It was already demonstrated that the mutation of the MIPS1 gene resulted in a reduction in the *myo*-inositol level and concomitantly a decrease of the raffinosaccharide level, and an increase of the sucrose level in LPA line LR33 seeds (*3*). In this study, we found that the raffinose and stachyose concentrations in *Gm-lpa*-TW-1 were significantly decreased. Since *Gm-lpa*-TW-1 also resulted from a mutation of the MIPS1 gene (*4*), such simultaneous reduction of the raffinosaccharide level and increase of the sucrose concentration are not unexpected. No significant and consistent differences were detected between Zhechun #3 and its mutant, implying that the LPA mutation did not disturb the metabolism of oligosaccharides. The increase of sucrose and decrease of raffinosacchride content presents an added value of the *Gm-lpa*-TW-1 mutation, particularly when it is used for vegetable soybean breeding.

There has been enormous interest in soybean isoflavone, particularly as nutraceutical and antioxidant agents (*25*). It has been well demonstrated that there are strong genotypic and environmental effects on the concentration of individual and total isoflavones (*26*, *27*). Substantial differences of isoflavone concentrations were also observed in this study, both between WT varieties and seeds of the same genotype produced in different locations/seasons (**Table 4**). Apart from that, we observed that the LPA mutant ZC-M consistently had isoflavone concentrations greater than its parent Zhechun #3 across all three environments (**Table 4**).

The differences between a mutant and its parent could result from different causes. First, and most often, it is due to the pleiotropic effect of a mutation. For example, the mutations in the MIPS1 gene could affect other traits associated in a common pathway, e.g., the raffinose and phytic acid discussed above. In such a situation, the differences between a mutant and its parent should remain in their progeny, for example, between the LPA and WT lines in the study. Our results showed that the LPA lines had on average remarkably higher isoflavone concentrations than WT lines (**Table 5**), suggesting that the high isoflavone concentration of ZC-M is possibly due to the LPA mutation. Another possibility that cannot be excluded is that there could be another linked or unlinked mutation that resulted in the higher isoflavone levels.

Several quantitative trait loci (QTLs) for total and individual isoflavone concentrations have been mapped to various linkage groups, i.e., A1, B1, B2, D1a+Q, H, K, and N (*28*, *29*), demonstrating the complexity of its genetic control. Interestingly, the two QTLs for genistein and daidzein are located on LG B2 in a region similar to that of the ZC-M LPA mutation (*4*); however, the effects of the two QTLs seem quite minor, with LOD 2.0 and 2.9, respectively, implying that they should not be the major causative factor leading to the significant isoflavone concentration differences between ZC-M and its parent. Cheng et al. (*27*) also reported that the genetic polymorphisms at the two isoflavone synthase (IFS) gene loci are associated with isoflavone concentration, but no clear mechanism was proposed.

Since there were also a few LPA lines having isoflavone concentrations comparable with or even lower than the highest concentrations of WT lines, it is difficult to make a sound conclusion of the exact cause that leads to the elevated isoflavone concentration in ZC-M. Further studies using subsequent generations from the same population that was used in this study and a larger population could verify whether the concurrence of low phytic acid and high isoflavone concentration is truly due to the pleitropic effect of the LPA mutation in ZC-M or to other yet unknown reasons. If it were so unveiled, it would be also intriguing to uncover the biological mechanism underpinning the association of the biosynthesis and regulation of phytic acid and isoflavones.

We previously reported that the two LPA mutations do not exert any negative effect on agronomic traits including seed viability (*4*) and mineral cations of nutritional value (*30*), and with the findings of this study for nutritional traits, it is now clear that both LPA mutations can be used in commercial breeding programs for developing high nutrition soybean varieties. Particularly, the high sucrose concentration in *Gm-lpa*-TW75-1 would add special flavor to soybean food when used as vegetable soybean. Furthermore, combining the two mutations into a single variety would develop soybean feed that has higher P availability and carbohydrate digestibility. Therefore, the two LPA mutant genes should be valuable genetic resources for breeding yield competitive and high quality soybean varieties.

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