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ARTICLE

Metabolite Profiling of Two Novel Low Phytic Acid (*lpa*) Soybean Mutants

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A GC-based approach was applied to compare the metabolite profiles of two low phytic acid (*lpa*) soybean mutants and their respective wild-types. The *lpa* mutants (*Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2) were grown together with the wild-types (Taiwan 75 and Zhechun no. 3) in three and four field trials, respectively. HPLC analysis revealed a phytic acid reduction of −53% for *Gm-lpa*-TW75-1 and of −46% for *Gm-lpa*-ZC-2. For *Gm-lpa*-TW75-1, no accumulation of lower inositol phosphates was observed, whereas *Gm-lpa*-ZC-2 exhibited significantly increased contents of the lower inositol phosphates $InsP_3$, $InsP_4$, and $InsP_5$ compared to the corresponding wild-type. The metabolite profiling revealed that compared to the wild-types, 40% (*Gm-lpa*-TW75-1) and 21% (*Gm-lpa*-ZC-2) of the detected peaks were statistically significantly different in the *lpa* mutants grown at one field trial. However, the majority of these differences were shown to be related to environmental impact and natural variability rather than to the mutation event. Identification of consistent metabolic changes in the *lpa* mutants revealed decreased contents of *myo*-inositol, galactinol, raffinose, stachyose, and the galactosyl cyclitols galactopinitol A, galactopinitol B, and fagopyritol B1 compared to the wild-type. These consistently pronounced changes in *Gm-lpa*-TW75-1 confirmed the suggested mutation target. Consideration of the metabolic changes observed for *Gm-lpa*-ZC-2 (accumulation of lower inositol phosphates and increased *myo*-inositol contents) indicated a mutation event affecting the latter biosynthetic steps leading to phytic acid. The study demonstrated the applicability of metabolite profiling for the detection of changes in the metabolite phenotype induced by mutation breeding and its power in assisting in the elucidation of mutation events.

KEYWORDS: Metabolite profiling; low phytic acid soybean; *Glycine max* L. Merr.; *myo*-inositol; raffinose oligosaccharides; galactosyl cyclitols

INTRODUCTION

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate, $InsP_6$) occurs ubiquitously in crops and represents the major storage form of phosphorus in mature seeds (*1*). In soybean, phytic acid contents from 1.3 to 4.6% have been reported (*2*, *3*). Phytic acid is considered to be an antinutrient in food and feed. It may chelate divalent cations such as Ca^{2+}, Fe^{2+}, and Zn^{2+} and thus limit the bioavailability of these nutritionally important minerals. During the past years, various crops, for example, rice, maize, barley, and wheat, have been developed with lowered contents of phytic acid (*4*). For soybean, low phytic acid (*lpa*) crops have been produced by genetic engineering (*5*, *6*) and by mutation breeding, for example, through chemical mutagenesis (*7*, *8*) and γ-irradiation (*9*).

Different mutation targets have been shown to be responsible for the *lpa* phenotype in soybean mutants (*10*). A single base change in the *myo*-inositol 1-phosphate synthase (MIPS1) gene resulted in a 50% decreased phytic acid content in soybean mutant LR33 accompanied by a nearly molar equivalent increased level of inorganic phosphorus (P_i) compared to the wild-type (*8*). At least one putative multidrug resistance-associated protein (MRP) gene has been identified as mutation target in the *lpa* soybean mutant CX1834 (M153) (*11*). Similar to LR33, this mutant showed a molar equivalent increase in P_i, whereas the phytic acid content was decreased by 55% (*7*).

Low phytic acid crop mutants are typically selected and classified on the basis of their increased levels of P_i. However, these induced mutations were also shown to result in further metabolic changes in crops. For LR33 soybeans, targeted analysis revealed decreased levels of *myo*-inositol, raffinose, and stachyose, whereas the sucrose content was increased (*8*). For high P_i lines derived from M153 soybeans, levels of sucrose, raffinose, and stachyose were reported to be unchanged compared to the parental line (*12*).

Metabolite profiling techniques aspire to provide a comprehensive picture of the metabolites present in a biological system (*13*, *14*). Recently, a metabolite profiling study on two *lpa* rice mutants demonstrated the applicability of this approach to detect metabolic differences between the *lpa* mutants and the corresponding wild-type (*15*). Consideration of these differences in

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light of the pathways involved in the biosynthesis of phytic acid allowed assumptions on the mutation targets in these mutants, which were shown to agree with molecular mapping and gene cloning results (*16*, *17*).

The objects of this investigation were two novel *lpa* soybean mutants (*Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2) developed by γ-irradiation (*9*). The phytic acid phosphorus reduction of *Gm-lpa*-TW75-1 was shown to result from a two base pair deletion in the MIPS1 gene. However, the sequence change is different from that in LR33. Similar to LR33 and M153, this *lpa* soybean mutant exhibited significantly increased P_i levels. In contrast to previously described *lpa* soybean mutants, the phytic acid reduction in *Gm-lpa*-ZC-2 is accompanied by an accumulation of lower inositol phosphates (*9*). However, the genetic background of this mutation has not been clarified fully. The aim of the present study was to compare the metabolic profiles of these two *lpa* soybean mutants with the corresponding wild-types by means of metabolite profiling to (i) demonstrate the applicability of metabolite profiling for the detection of changes in the metabolite phenotype induced by mutation breeding and (ii) confirm its suitability to assist in the elucidation of mutation events. To assess the environmental impact on the metabolite levels, soybean wild-types and mutants were grown at different field trials and the resulting metabolite profiles were subjected to multivariate and univariate statistical analyses.

MATERIALS AND METHODS

Chemicals. Internal standards (tetracosane, 5α-cholestan-3β-ol, phenyl-β-D-glucopyranoside, *p*-chloro-L-phenylalanine) and retention time standards (undecane, hexadecane, tetracosane, triacontane, octatriacontane) were purchased from Fluka (Taufkirchen, Germany). Reference compounds were obtained from VWR International (Darmstadt, Germany), Fluka (Taufkirchen, Germany), Sigma-Aldrich (Steinheim, Germany), Cognis (Illertissen, Germany), and Roth (Karlsruhe, Germany). All other reagents and solvents were of analytical grade.

Materials. *Wild-Type and lpa Soybean Mutants*. Low phytic acid mutant lines *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2 were generated through γ-irradiation of the wild-types Taiwan 75 and Zhechun no. 3, respectively, at the Irradiation Center of Zhejiang University, China. Low phytic acid soybean mutants were grown together with their corresponding wild-types in Hainan (China) in the winter/spring seasons of 2004/2005 and 2005/2006 and in the spring (April−July) and autumn (August−October) seasons in Hangzhou (China). For each field trial, the seeds from wild-type soybeans and *lpa* mutants, respectively, were bulked and sun-dried. The materials were not subjected to further postharvest treatments before analysis.

Sample Preparation. Freeze-dried soybean seeds were ground with a cyclone mill equipped with a 500 μm sieve (Cyclotec, Foss, Germany). The resulting flour was freeze-dried again for 48 h (Alpha 1-4 LSC, Christ, Germany) and stored at −18 °C until analysis.

Analysis of Inositol Phosphates. Isolation and separation of inositol phosphates were performed in accordance with previously described procedures (*15*). Quantification of $InsP_6$ was based on external calibration using standard solutions of phytic acid dodecasodium salt in the mobile phase (0.25−8.00 mg/mL). Contents of lower inositol phosphates ($InsP_5$, $InsP_4$, $InsP_3$) were quantified on the basis of the calibration curve of phytate considering correction factors for lower inositol phosphates reported in the literature (*18*).

Recoveries of $InsP_3$, $InsP_4$, $InsP_5$, and $InsP_6$ from soybean samples spiked with the technical phytic acid solution were 94 ± 0, 109 ± 5, 116 ± 3, and 101 ± 0% (mean ± SD, n = 3). On the basis of a signal-to-noise ratio of 3:1, limits of quantitation were calculated to be 0.13% for $InsP_3$, 0.11% for $InsP_4$, and 0.07% for $InsP_5$ and $InsP_6$.

Metabolite Profiling. *Preparation of Retention Time Standards and Internal Standards*. Retention time standard 1 was prepared by adding *n*-hexane solutions of undecane (1.5 mL, 2 mg/mL), hexadecane (2.5 mL, 1.5 mg/mL), tetracosane (4 mL, 1.5 mg/mL), and triacontane (4 mL, 1.5 mg/mL) to 15 mg of octatriacontane. Retention time standard 2 was prepared by adding 1.5 mL of *n*-hexane and *n*-hexane solutions of hexadecane (2.5 mL, 1.5 mg/mL), tetracosane (4 mL, 1.5 mg/mL), and triacontane (4 mL, 1.5 mg/mL) to 15 mg of octatriacontane.

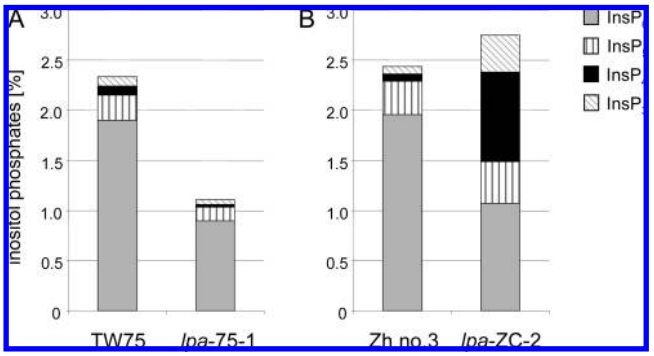

Figure 1. Mean contents and distribution of phytic acid ($InsP_6$) and lower inositol phosphates ($InsP_3$, $InsP_4$, $InsP_5$) in Taiwan 75 and *Gm-lpa*-TW75-1 (**A**) and Zhechun no. 3 and *Gm-lpa*-ZC-2 (**B**) grown at three and four locations, respectively.

Internal standard solution I was identical to retention time standard 1 (tetracosane was used as internal standard). A solution of 5α-cholestan-3β-ol in dichloromethane (0.3 mg/mL) was used as internal standard solution II. Phenyl-β-D-glucopyranoside in deionized water (0.8 mg/mL) and *p*-chloro-L-phenylalanine in deionized water (0.3 mg/mL) were used as internal standard solutions III and IV, respectively.

The extraction and fractionation of freeze-dried soybean flour as well as evaporation and silylation conditions for nonpolar and polar extracts were in accordance with previously described procedures for rice (*15*) with slight modifications. Briefly, lipids and polar compounds were extracted consecutively from 300 mg of flour with 4 mL of dichloromethane and a mixture of 10 mL of methanol and deionized water (80 + 20, v/v), respectively. One hundred microliters of each internal standard solution (I and II) was added to the nonpolar extract. After evaporation under vacuum to dryness, the residue was redissolved in 500 μL of methyl *tert*-butyl ether (MTBE), 300 μL of dry methanol, and 50 μL of sodium methylate (5.4 M in methanol). After reaction for 90 min at room temperature, 1 mL of dichloromethane and 2 mL of aqueous 0.35 M hydrochloric acid were added, and the mixture was vigorously shaken. The upper phase was discarded, and the lower phase containing the transmethylated lipids was evaporated to dryness under vacuum. Transmethylated lipids were separated by solid phase extraction (SPE) into a fraction containing fatty acid methyl esters (FAME) and hydrocarbons (fraction I) and a fraction containing minor lipids, for example, sterols and free fatty acids (fraction II). Therefore, the total lipids were redissolved in 250 μL of dichloromethane and transferred to the SPE cartridge (LiChrolut, VWR International, Germany). Fraction I was eluted with hexane/MTBE (100 + 2, v/v, 2 × 3 mL). Fraction II was eluted with hexane/MTBE (70 + 30, v/v, 2 × 3 mL).

Prior to further fractionation of the polar compounds, the polar extract was passed through a 0.45 μm filter (Whatman, Germany), and 250 μL of each internal standard solution (III and IV) was added. The dry residue of 1 mL of polar extract was redissolved in 300 μL of pyridine and silylated with 100 μL of trimethylsilylimidazole. Three hundred microliters of *n*-hexane and 300 μL of deionized water were added for selective hydrolysis of the silylated derivatives of organic acids and amino acids and to obtain a fraction in the hexane phase containing the stable silylated sugars and sugar alcohols (fraction III). To obtain a fraction containing organic acids and amino acids (fraction IV), a separate aliquot of 2 mL of polar extract was used. The dry residue was redissolved and heated for oximation in 300 μL of a solution of hydroxylammonium chloride in pyridine (2 mg/mL). After silylation with 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, 500 μL of *n*-hexane and 300 μL of deionized water were added to the silylated sample. The aqueous phase was evaporated to dryness. The residue was redissolved in 200 μL of acetonitrile, and 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was added for silylation.

The four fractions obtained were analyzed by capillary gas chromatography (GC-FID, GC-MS). GC-FID analysis was performed on a Focus GC (ThermoFinnigan, Austin, TX) equipped with a flame ionization detector (FID; 320 °C) using a DB-1, 60 m × 0.32 mm i.d. fused silica

capillary coated with a 0.25 μm film of polydimethylsiloxane (J&W Scientific, Folsom, CA). Hydrogen was used as the carrier gas (flow = 1.8 mL/min). Split injection (split flow = 27 mL/min) was performed at 250 °C. The column temperature was programmed from 100 to 320 °C (25 min hold) at 4 °C/min.

GC-MS analysis was performed on a Finnigan TraceGC ultra coupled to a quadrupole mass selective detector Finnigan Trace DSQ (ThermoFinnigan) using a factorFOUR VF-1 ms (60 m × 0.32 i.d. coated with a 0.25 μm film of polydimethylsiloxane (Varian, Darmstadt, Germany). The MS interface temperature was set to 320 °C and the

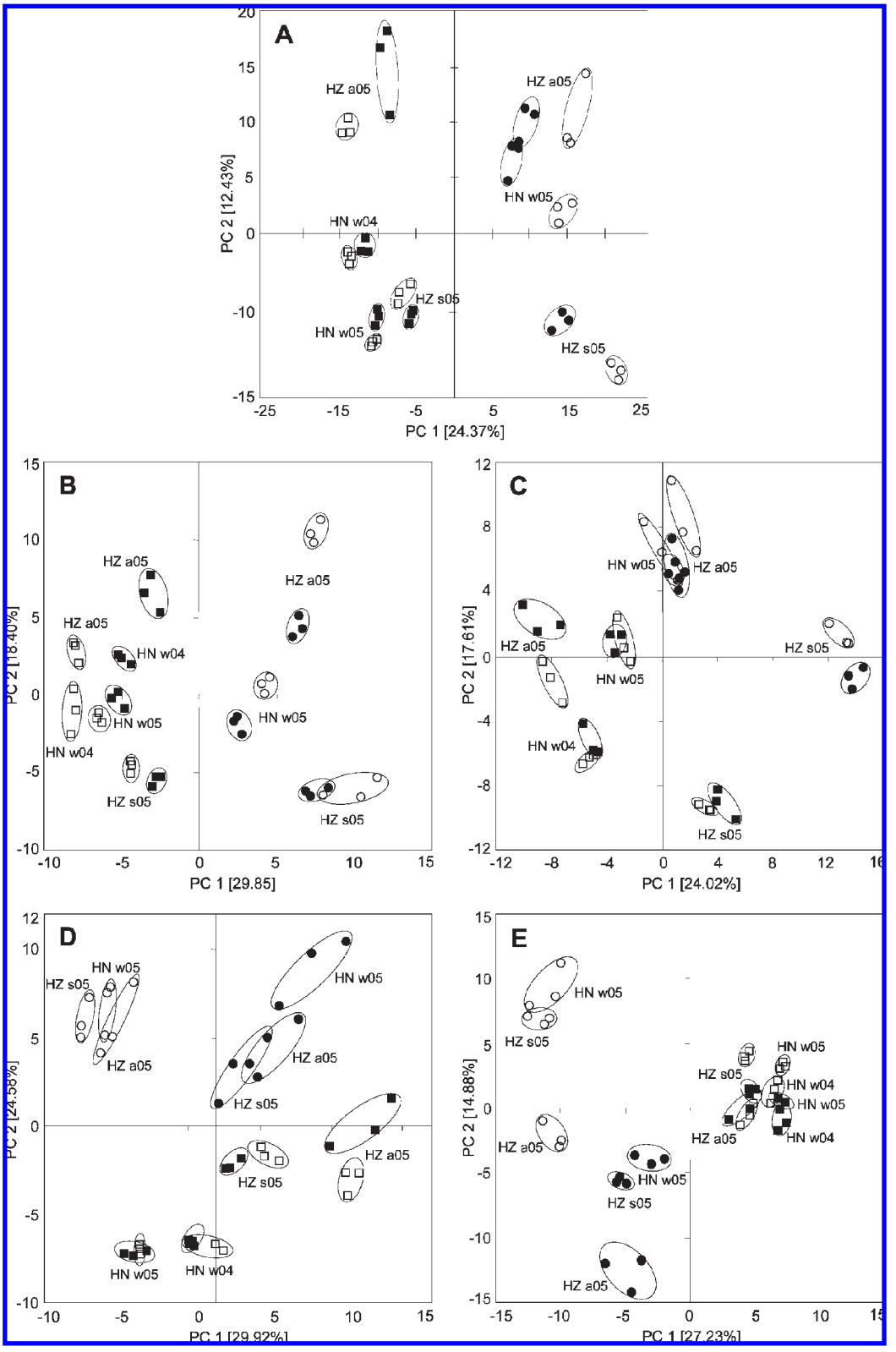

Figure 2. Principal component analyses (PCA) of standardized GC-FID metabolite profiling data from the combined fractions I−IV (**A**) and single fractions I (**B**), II (**C**), III (**D**), and IV (**E**) of the wild-type soybeans Taiwan 75 (●) and Zhechun no. 3 (■) and *lpa* mutants *Gm-lpa*-TW75-1 (○) and *Gm-lpa*-ZC-2 (□) grown at Hainan and Hangzhou in 2004/2005−2005/2006. HN w04 and w05, Hainan winter 2004 and 2005; HZ s05 and a05, Hangzhou spring 2005 and autumn 2005.

Table 1. Peak-Based Comparison of Chromatograms Obtained by Metabolite Profiling of Wild-Types (Taiwan 75 and Zhechun No. 3) and *Lpa* Mutants (*Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2) Grown at Three and Four Field Trials, Respectively

	field trial[a]								
	Hainan, w04/05		Hainan, w05/06		Hangzhou, s05		Hangzhou a05		
wild-type vs *lpa* mutant	total[b]	diff[c]	total[b]	diff[c]	total[b]	diff[c]	total[b]	diff[c]	consistent diff[d]
TW75 vs *lpa*-75-1			168	69	181	65	165	70	23
Zh no. 3 vs *lpa*-ZC-2	166	27	164	55	157	13	167	43	3

[a] Hainan, w04/05, and Hainan, w05/06, winter seasons; Hangzhou, s05, spring season; and Hangzhou, a05, autumn season. [b] Number of peaks included for comparison in fractions I−IV (peak height > 500 μV). [c] Number of peaks statistically significantly different between wild-type and mutant line in fractions I−IV ($p < 0.05$). [d] Number of peaks statistically significantly different between wild-type and mutant line at all field trials.

Table 2. Peak Responses of Compounds Found To Be Statistically Significantly and Consistently Different between Taiwan 75 and *Gm-lpa*-TW75-1 and between Zhechun No. 3 and *Gm-lpa*-ZC-2 Grown at Three and Four Field Trials, Respectively

		response (mV)			
fraction	compound	Taiwan 75	*Gm-lpa*-TW75-1	ratio	identification[a]
I	heptadecene	1.40 ± 0.05	2.15 ± 0.10	1.54	B
III	glycerol	0.65 ± 0.17	1.63 ± 0.38	2.51	A
	sorbitol	0.74 ± 0.09	1.42 ± 0.05	1.92	A
	myo-inositol	6.21 ± 1.66	0.76 ± 0.14	0.12	A
	sucrose	200 ± 31	306 ± 25	1.53	A
	galactopinitol A	8.78 ± 2.85	0.67 ± 0.18	0.08	C, E
	galactopinitol B	9.21 ± 2.21	0.83 ± 0.19	0.09	C, E
	fagopyritol B1	4.09 ± 1.00	1.15 ± 0.54	0.28	C, E
	galactinol	0.68 ± 0.09	bld[b]		A
	galactose derivate	1.66 ± 0.19	0.69 ± 0.08	0.41	E
	galactose derivate	2.35 ± 0.43	0.43 ± 0.06	0.18	E
	raffinose	63 ± 5	16 ± 1	0.26	A
	ciceritol	1.85 ± 0.18	bld		D, E
	unknown[c]	1.41 ± 0.21	bld		
	stachyose	37 ± 2	0.35 ± 0.09	0.01	A
IV	phosphoric acid	37 ± 8	220 ± 44	5.93	A
	valine	1.29 ± 0.25	3.71 ± 0.56	2.88	A
	leucine	1.47 ± 0.38	4.08 ± 0.95	2.78	A
	serine	0.63 ± 0.15	1.78 ± 0.48	2.83	A
	β-alanine	0.54 ± 0.09	1.56 ± 0.32	2.88	A
	asparagine	1.05 ± 0.24	5.72 ± 2.34	5.44	A
	citrulline	0.70 ± 0.12	2.23 ± 0.55	3.17	A
	tyrosine	1.44 ± 0.84	0.49 ± 0.14	0.34	A
		Zhechun no. 3	*Gm-lpa*-ZC-2		
III	*myo*-inositol	2.19 ± 0.28	4.29 ± 0.48	1.96	A
IV	phosphoric acid	45 ± 7	94 ± 12	2.11	A
	syringic acid	2.37 ± 0.94	3.72 ± 1.25	1.57	A

[a] Identification according to A, mass spectrometric data and retention time of reference compound; B, NIST 02 MS library; C, Schweizer et al. (*25*) and Schweizer and Horman (*26*); D, Quemener and Brillouet (*27*); and E, MS data (see **Figure 4**). [b] Below the limit of detection. [c] No MS data available.

source temperature to 250 °C. Full-scan mass spectra were recorded at an electron energy of 70 eV within a scan range of 40−700 mu at a scan rate of 2.5 scans/s. Helium was used as the carrier gas at a constant flow of 1 mL/min. The chromatographic conditions were as described for GC-FID analysis.

Soybean constituents were identified by comparing retention times and mass spectra with those of trimethylsilylated reference compounds and by comparing mass spectra with the entries of the mass spectra library NIST02.

Statistical Analysis. For each field trial, one sample from the bulked seed materials was analyzed in triplicate. GC-FID data were acquired and integrated using Chrom-Card 2.3 (Thermo Electron, Italy). Peak heights and retention times were exported to Chrom*pare* 1.1, a software tool developed for comparative analysis of metabolite profiling data (http://www.chrompare.com) (*19*). Chrom*pare* automatically corrects retention time shifts on the basis of retention time standards (consolidation) and standardizes peak heights on the basis of internal standards added prior to the fractionation process. Comparison of metabolite profiles was performed by comparison of mean standardized peak heights based on triplicate analysis. Principal component analysis (PCA) was performed using XLSTAT 7.5.2 (Addinsoft, France).

RESULTS AND DISCUSSION

Analysis of Inositol Phosphates. Mean contents of phytic acid and of lower inositol phosphates ($InsP_3$, $InsP_4$, $InsP_5$) determined in the wild-types (Taiwan 75, Zhechun no. 3) and in the *lpa* mutants (*Gm-lpa*-TW75-1, *Gm-lpa*-ZC-2) grown side by side at different locations are shown in **Figure 1**. On average, the degree of phytic acid reduction compared to the wild-type was slightly higher for *Gm-lpa*-TW75-1 (−53%) than for *Gm-lpa*-ZC-2 (−46%). For *Gm-lpa*-TW75-1, no accumulation of lower inositol phosphates was observed (**Figure 1A**), whereas *Gm-lpa*-ZC-2 exhibited significantly increased contents of $InsP_3$, $InsP_4$, and $InsP_5$ (**Figure 1B**). This finding agrees with results obtained for this mutant in a previous field trial (*9*). The sum of the inositol phosphates $InsP_3$−$InsP_6$ appears to be slightly higher in the mutant than in the wild-type (**Figure 1B**). However, this difference might be due to the quantification of lower inositol phosphates based on a calibration curve of phytate only using correction factors for the lower $InsP_s$ as described in the literature (*18*).

Metabolite Profiling. Freeze-dried soybean flour was subjected to a metabolite profiling procedure that resulted in four fractions containing fatty acid methyl esters and hydrocarbons (fraction I), free fatty acids, fatty alcohols, and sterols (fraction II), sugars and sugar alcohols (fraction III), and amino acids, organic and inorganic acids, and amines (fraction IV). The procedure was in accordance with a methodology reported previously for rice (*15*). Adaptation of the extraction and fractionation protocols to the soybean matrix required slight modifications. The higher contents of total lipids and polar compounds in soybeans (*21*) was taken into account by decreasing the amount of freeze-dried flour subjected to the initial extraction and by adjusting the amount of polar extract used for fractionation. The fractions were analyzed by capillary gas chromatography. GC-FID analysis resulted in the detection of 613 distinct peaks in the four fractions from the two *lpa* mutants and wild-types.

Multivariate Analysis. A PCA of the standardized and consolidated GC-FID metabolite profiling data was conducted to see if the soybean samples could be classified according to cultivar, type of mutation, and growing location/season. Scores plots obtained for the combined fractions I−IV and for each fraction are shown in **Figure 2**.

For the scores plot of the combined fractions I−IV, the first two PCs explained 37% of the total variance (**Figure 2A**). There

are differentiations according to the growing locations/seasons for the wild-types as well as for the corresponding mutants. The mutant *Gm-lpa*-TW75-1 is separated consistently from the wild-type Taiwan 75 at all field trials. In contrast, the variance between the mutant *Gm-lpa*-ZC-2 and the wild-type Zhechun no. 3 is less pronounced, resulting in a clear separation at only one field trial.

The same phenomenon is reflected by the scores plots of the single fractions III (**Figure 2D**) and IV (**Figure 2E**). The distinct and consistent separations of *Gm-lpa*-TW75-1 and the wild-type Taiwan 75 indicate a strong influence of the mutation on the polar metabolite profiles in this mutant. In contrast to the polar fractions, no major shift was observed in the nonpolar fractions (**Figure 2B**,**C**) for this mutant compared to wild-type. Instead, a location-dependent separation of the side-by-side grown mutant *Gm-lpa*-TW75-1 and the corresponding wild-type was observed that indicates a more pronounced environmental impact rather than mutational effects on the lipid profile.

Assessment of the scores plots of polar and nonpolar fractions for *Gm-lpa*-ZC-2 and Zhechun no. 3 exhibited no major differences between the *lpa* mutant and the corresponding wild-type grown at one location. However, similar to the observations made for *Gm-lpa*-TW75-1 in the nonpolar fractions, a considerable effect of the growing location was detected for both nonpolar and polar fractions. In addition to an influence of the growing location, a significant effect was also observed for the different growing seasons. *Lpa* mutants and corresponding wild-types grown in the spring season of 2005 at Hangzhou were separated from the rest of the samples in the two nonpolar fractions (**Figure 2B**,**C**). An explanation for this could be the subtropical climatic condition at Hangzhou during this season. It was shown that both *lpa* mutants and wild-types had a lower seedling emergence when they were produced in the spring season at this location (*9*). A low phytate-related impairment on seedling emergence has been also shown for LR33- and M153-derived *lpa* lines, especially when the plants were grown in a (sub)tropical environment (*22*, *23*).

Univariate Analysis. The assessment of the corresponding PCA loadings is one of the approaches suitable to determine the metabolites responsible for the clustering seen in **Figure 2**. However, these loadings would also reflect metabolic changes due to both the mutation events and the environmental impact (locations/seasons). To identify the compositional differences that are solely caused by the mutations, the assessment of the metabolite profiling data was performed by univariate analysis. The objective was to detect metabolic differences between wild-types and *lpa* mutants which are consistent, that is, statistically significant, at all field trials. Therefore, GC-FID data (peak heights and corresponding retention times) were imported to Chrom*pare*, a software tool for automatic comparative analysis of metabolite profiling data (*19*). Results from the comparative analysis of fractions I−IV obtained by metabolite profiling of the *lpa* mutant lines and the corresponding wild-types are shown in **Table 1**. For the field trials analyzed, on average, 171 peaks were included for the comparison of *Gm-lpa*-TW75-1 versus Taiwan 75 and 164 peaks for *Gm-lpa*-ZC-2 versus Zhechun no. 3. For *Gm-lpa*-TW75-1 68 (40%) and for *Gm-lpa*-ZC-2 35 (21%) statistically significantly different peaks were found compared to the corresponding wild-type at the field trials. The percentages of statistically significant differences in metabolites between the two *lpa* mutants and wild-types for each field trial are in the same order of magnitude as those determined for comparable GC-based metabolite profiling studies on *lpa* mutants of maize (*24*) and rice (*15*).

Assessment of statistically significantly differences between *lpa* soybean mutants and wild-types revealed that only some were consistently present at all analyzed field trials. This observation is in agreement with results obtained for *lpa* rice mutants, confirming the importance of environmental impact and natural variability on plant metabolite profiles (*15*).

The differences seen in the PCA scores plots regarding the separation of wild-types and mutants were also reflected in the number of consistent differences determined by the univariate analysis. For *Gm-lpa*-TW75-1, 23 of the statistically significantly

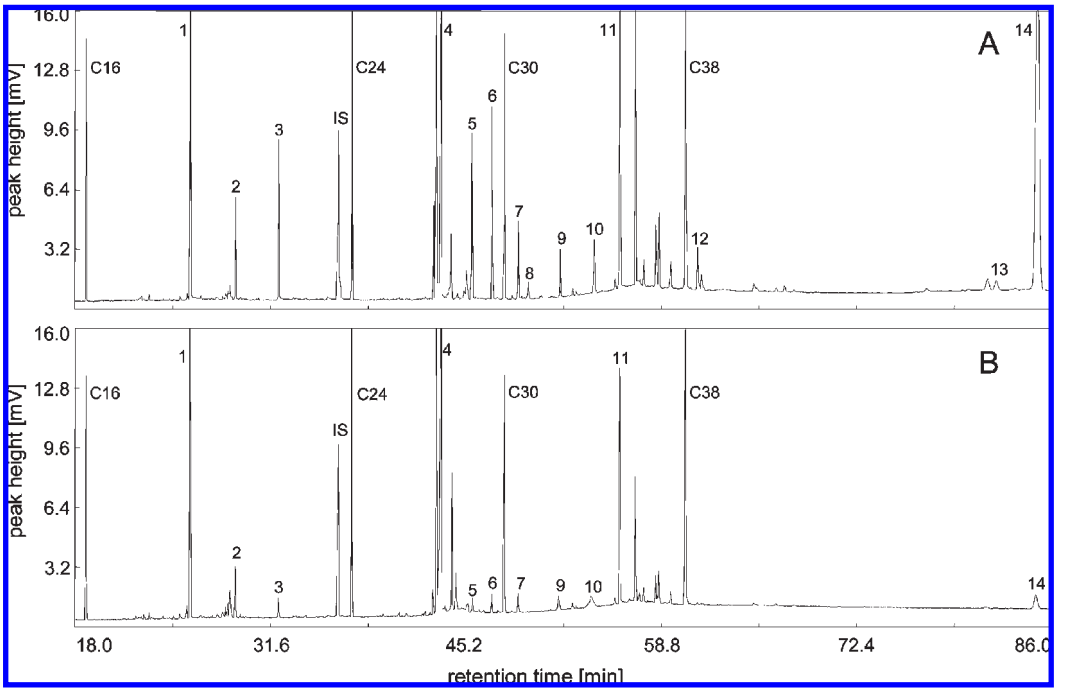

Figure 3. GC-FID chromatograms of fraction III obtained for the wild-type Taiwan 75 (**A**) and the *lpa* mutant *Gm-lpa*-TW75-1 (**B**) grown in spring season 2005 in Hangzhou. Peaks 1−14: TMS derivatives of D-pinitol (1), *chiro*-inositol (2), *myo*-inositol (3), sucrose (4), galactopinitol A (5), galactopinitol B (6), fagopyritol B1 (7), galactinol (8), nonidentified galactose derivatives (9, 10), raffinose (11), ciceritol (12), unknown (13), and stachyose (14). Peaks C16 (hexadecane), C24 (tetracosane), C30 (triacontane), C38 (octatriacontane): retention time standards. IS: internal standard phenyl-β-D-glucopyranoside.

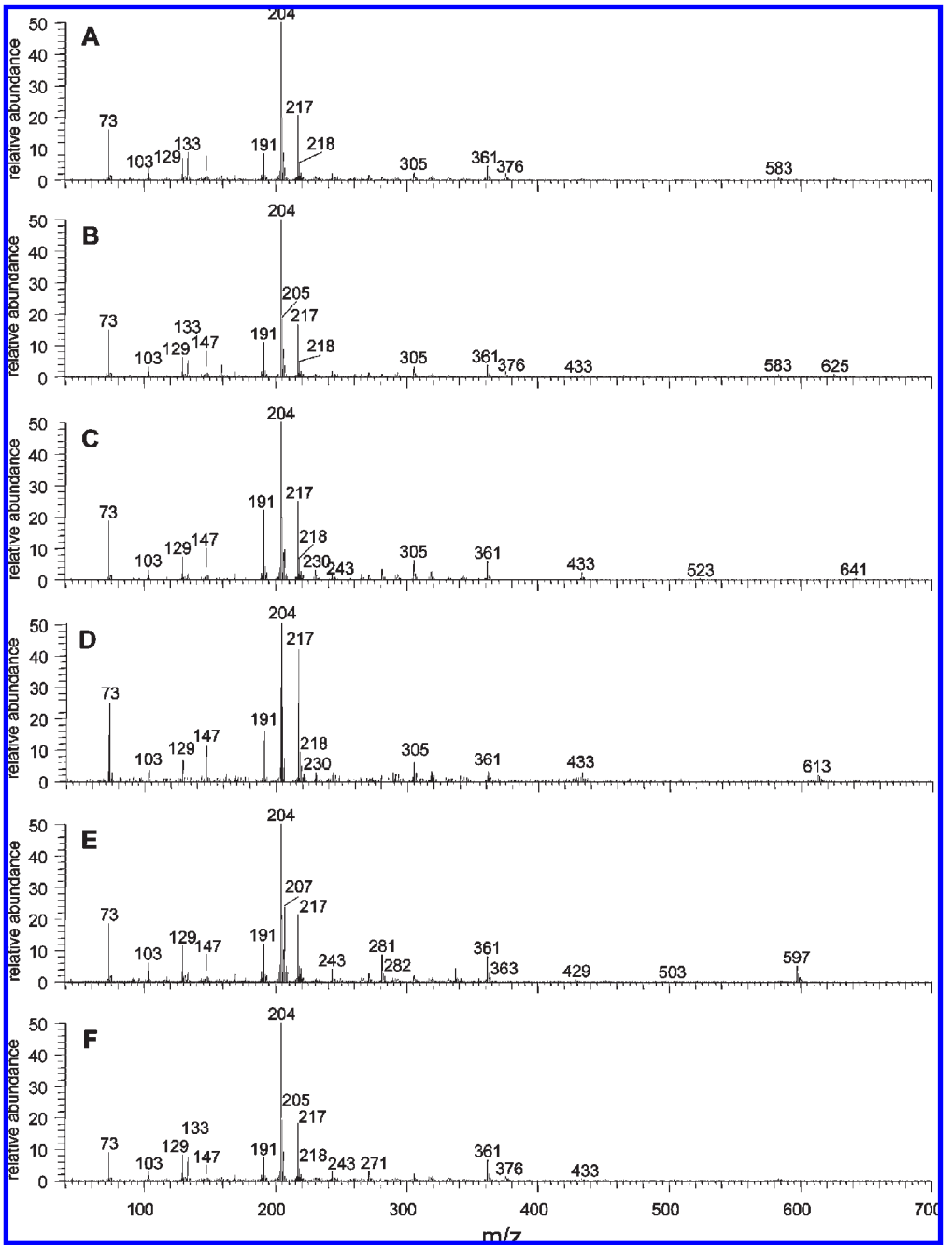

Figure 4. Electron impact mass spectra of tentatively identified TMS derivatives of galactopinitol A (**A**), galactopinitol B (**B**), fagopyritol B1 (**C**), ciceritol (**D**), and nonidentified galactose derivatives (**E**, **F**).

differences compared to the wild-type were found to be present consistently at all field trials, whereas only 3 were found to be different consistently between *Gm-lpa*-ZC-2 and the wild-type. Significantly and consistently different compounds between the wild-types and *lpa* mutants are given in **Table 2**. The three consistently different compounds in *Gm-lpa*-ZC-2 were present in fractions III and IV and were identified as trimethylsilyl (TMS) derivatives of phosphate, *myo*-inositol, and syringic acid. Their amounts were increased significantly compared to the wild-type. The majority of the 23 consistent differences between the *lpa* mutant *Gm-lpa*-TW75-1 and its wild-type were detected in fraction III. This point is illustrated by the GC-FID chromatograms of fractions III obtained from the wild-type and *Gm-lpa*-TW75-1 grown in Hangzhou (spring season 2005) (**Figure 3**). TMS derivatives of the significantly changed compounds glycerol, sorbitol, *myo*-inositol, sucrose, galactinol, raffinose, and stachyose were identified by comparing mass spectrometric data and retention times to those of authentic reference compounds. In addition, the galactosyl cyclitols galactopinitol A, galactopinitol B, fagopyritol B1, and ciceritol were identified tentatively on the basis of comparison of their mass spectra (**Figure 4A−D**) and their retention behaviors to those described for these galactosyl cyclitols in the literature (*25−27*). Three other consistently different peaks detected in fraction III were not identified. However, MS data of two of the peaks indicate that these compounds are also galactose derivatives (**Figure 4E**,**F**). In legumes, a broad range of cyclitols and galactosyl derivatives has been reported (*28−31*).

The mutant *Gm-lpa*-TW75-1 exhibited significantly increased levels of glycerol, sorbitol, and sucrose, whereas the amounts of

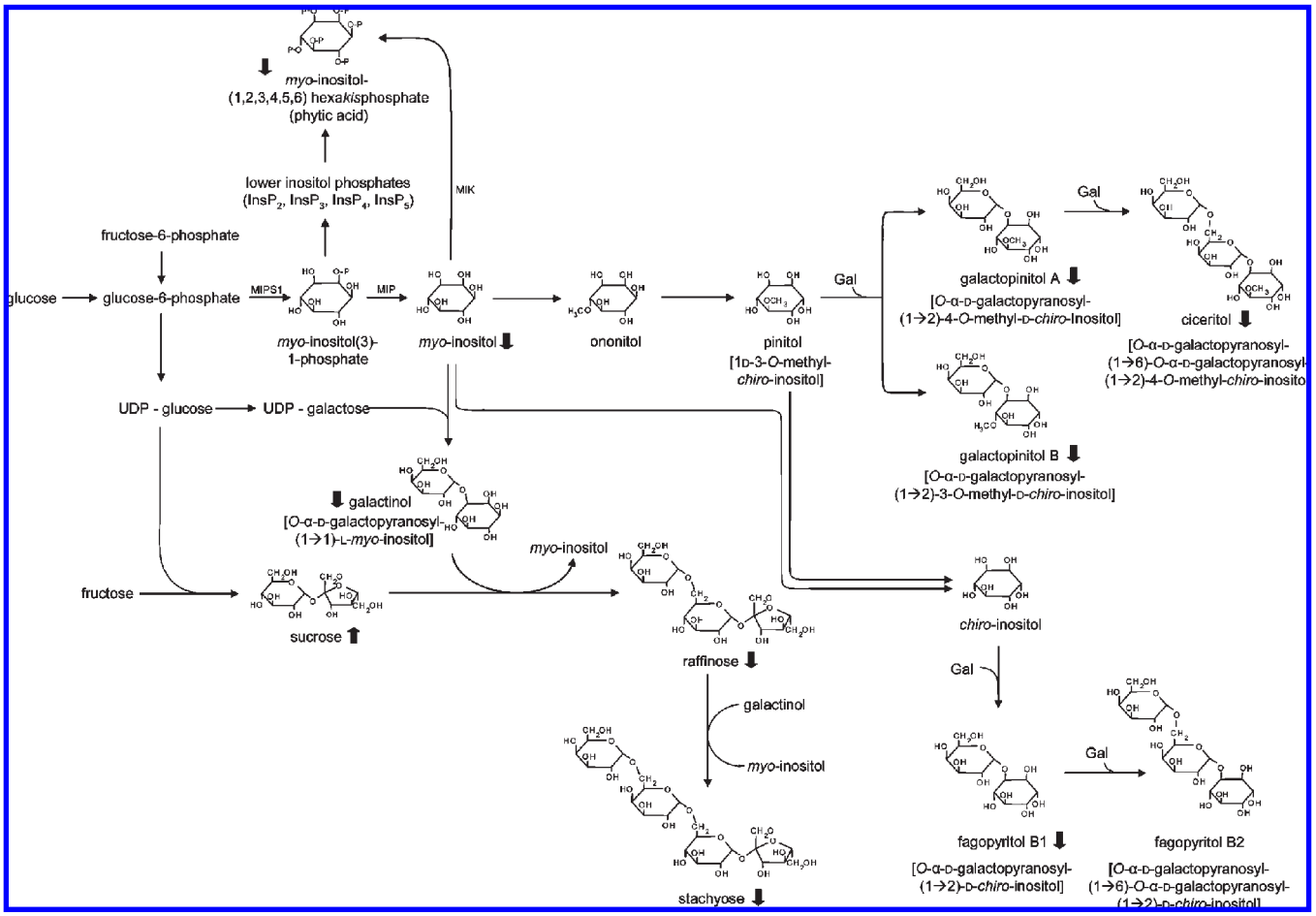

Figure 5. Link between the biosynthetic pathways leading to phytic acid, raffinose oligosaccharides, and galactosyl cyclitols. MIPS1, 1D-*myo*-inositol 3-phosphate synthase; MIP, *myo*-inositol monophosphatase. Arrows indicate statistically significantly metabolic changes between the *lpa* mutant *Gm-lpa*-TW75-1 and the wild-type Taiwan 75.

all other compounds found to be consistently different in fraction III were shown to be statistically significantly decreased in the mutant.

Biogenetic Aspects. The biosynthesis of phytic acid starts with the conversion of D-glucose 6-phosphate to 1D-*myo*-inositol 3-phosphate [Ins(3)P_1] through D-*myo*-inositol 3-phosphate synthase (MIPS1), followed by kinase-catalyzed phosphorylation to higher homologues (*4*) (**Figure 5**). In addition, *myo*-inositol formed by dephosphorylation of Ins(*3*)P_1 is considered to be an important intermediate in the pathway leading to phytic acid (*4*, *32*).

Gm-lpa-TW75-1. The *lpa* soybean mutant *Gm-lpa*-TW75-1 is characterized by the absence of lower inositol phosphates. The reduction in phytic acid content is accompanied by a molar equivalent increase of P_i (*9*). Therefore, the observed significant increase in free phosphate was expected. In addition to the altered phosphate content, the comparative metabolite profiling of *Gm-lpa*-TW75-1 and Taiwan 75 revealed significantly decreased contents of *myo*-inositol, oligosaccharides, and the tentatively identified galactosyl cyclitols in the *lpa* mutant, whereas sucrose was shown to be increased. As shown in **Figure 5**, the biosynthetic pathways of phytic acid, raffinose oligosaccharides, and galactosyl cyclitols are closely linked. Changes observed for these sugars in the metabolite profiles are in excellent agreement with phytic acid reduction in this mutant due to a lesion in the MIPS1 gene (*9*).

Gm-lpa-ZC-2. Of the various consistent changes observed for metabolites in *Gm-lpa*-TW75-1 compared to the wild-type, metabolite profiling of the mutant *Gm-lpa*-ZC-2 revealed consistent increases only of the contents of phosphate (the intended effect), of syringic acid, and of *myo*-inositol compared to wild-type. Together with the accumulation of lower inositol phosphates, the metabolite profiling data clearly show that the phytic acid reductions in the two *lpa* soybeans investigated in this study are based on different mutations. Increased levels of *myo*-inositol have also been reported for *lpa* mutants of maize (*33*) and barley (*34*), which also showed accumulation of lower inositol phosphates (*35*, *36*). For *lpa*2 maize, a mutation in an inositol phosphate kinase (ZmIpk) gene was shown to be responsible for the observed phenotype (*33*). The mutation target in *Gm-lpa*-ZC-2 has been located by molecular mapping (*9*); however, identification of the associated enzyme(s) is still pending. Accumulation of lower inositol phosphates and increase of *myo*-inositol suggest that this mutation results from a lesion in one of the inositol phosphate kinases involved in the latter steps of the biosynthesis of phytic acid.

Metabolite profiling and inositol phosphate analysis of the two *lpa* soybean mutants revealed distinct differences between *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2. The majority of statistically significantly differences between *lpa* mutants and wild-types were shown to be related to environmental impact and natural variability rather than to the mutation event. The consistent metabolic changes observed in *Gm-lpa*-TW75-1 confirmed the suggested mutation target (*9*) and demonstrate the power of metabolite profiling in assisting in the elucidation of mutation events.

The unbiased and nontargeted screening of a broad spectrum of metabolites also increases the probability of detecting effects

not intended by the mutation process and thus may contribute to a nutritional quality and safety analysis of the induced mutants. The consistent metabolic changes observed in the *lpa* soybean mutants compared to the wild-types have to be considered when the nutritional value of the two mutants is assessed. The decreased contents of oligosaccharides in *Gm-lpa*-TW75-1 would be a highly desirable trait as they may cause flatulence when consumed by humans (*37*). Oligosaccharides cannot be hydrolyzed in the intestine of humans due to the absence of the enzyme α-galactosidase. For this reason, galactosyl cyclitols are also being discussed to produce flatulence (*29*).

From an agronomic point of view, such nutritional improvements would also have to be considered in light of crop performance. The yields of the soybean lines were not evaluated in the present study. Previous field trials showed that the wild-type Taiwan 75 had a higher grain yield (~40%) than its mutant *Gm-lpa*-TW-75-1, whereas no significant yield differences were observed for Zhechun no. 3 and *Gm-lpa*-ZC-2 (*20*).

Except for tyrosine, levels of valine, leucine, serine, β-alanine, asparagine, and citrulline were significantly increased in *Gm-lpa*-TW75-1 compared to the corresponding wild-type. However, at this point it cannot be finally concluded whether these observations result from the intended mutation, from pleiotropic effects, or from further mutations independent of the primary mutation event. A single environmental impact seems to be unlikely as the observed metabolic changes were consistently present at three different field trials within two seasons. A targeted analysis of amino acids in *Gm-lpa*-TW75-1 and the wild-type grown in the spring season 2004 at Hangzhou revealed no statistically significantly amino acid concentrations (*38*). However, amino acid concentrations were also slightly increased. Therefore, further studies on the amino acid contents in the wild-type and *lpa* mutant would be necessary.

Another result that deserves further attention is the consistent increase in syringic acid observed in *Gm-lpa*-ZC-2. Considering the important roles of phenolic compounds in soybean, it might be useful to confirm whether this effect is also observed in other field trials and whether this increase might be an indicator for other changes among the nutritionally important soybean phenolics.

For all effects seen in this study, it has to be kept in mind that for each field trial only one sample from the bulked seed materials was analyzed in triplicate. That means that despite the investigation of soybeans from different locations and different seasons, the "environmental" treatment has not been fully replicated. Extended analysis of further field trials might be useful to substantiate the mutation effects seen in this study in light of natural variability.

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