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

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Two low phytic acid (*lpa*) rice mutant lines, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2, were grown together with their parent wild-type variety Xiushui 110 in four field trials. HPLC analysis of inositol phosphates in the seeds produced demonstrated that compared to the wild-type, the reduction in phytic acid content in *Os-lpa*-XS110-1 (−46%) was more pronounced than that in *Os-lpa*-XS110-2 (−23%). Lower inositol phosphates ($InsP_3$, $InsP_4$, $InsP_5$) were not detected in the mutants. The *lpa* mutants and the wild-type rice were subjected to comparative metabolite profiling by capillary gas chromatography. On average, 34% (*Os-lpa*-XS110-1) and 42% (*Os-lpa*-XS110-2) of the detected peaks were statistically significantly different between wild-type and mutants. However, only a few of these differences could be consistently observed for all field trials. Identification and quantification of the consistently different metabolites revealed that contents of *myo*-inositol and raffinose were increased in *Os-lpa*-XS110-1 but decreased in *Os-lpa*-XS110-2 compared to the wild-type. In addition, *Os-lpa*-XS110-1 exhibited increased levels of galactose and galactinol. Consideration of these metabolic changes in light of the routes involved in the biosynthesis of phytic acid indicated a disturbance in the early biosynthetic pathway of phytic acid in *Os-lpa*-XS110-2 (similar to the *lpa*-1 type mutation in maize) and a mutation event affecting phosphorylation of *myo*-inositol in *Os-lpa*-XS110-1 (similar to the *lpa*-3-type mutation).

KEYWORDS: Low phytic acid mutants; rice; metabolite profiling; *Oryza sativa* L.; phytic acid; *myo*-inositol; raffinose; *myo*-inositol hexakisphosphate; galactinol; *myo*-inositol kinase

INTRODUCTION

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate or $InsP_6$) is the major storage form of phosphorus in mature seeds or grains. Typically, between 65 and 85% of seed total phosphorus is found in this compound (*1*). In beans phytic acid contents up to 9% have been described (*2*). The contents in cereals vary from 0.6 to 2.2%; amounts reported in brown rice range from 0.6 to 1.1% (*2, 3*).

The first step in the biosynthesis of phytic acid is the conversion of D-glucose 6-phosphate to 1D-*myo*-inositol 3-phosphate [Ins(3)P_1] catalyzed by 1D-*myo*-inositol 3-phosphate synthase (MIPS) (*4, 5*). The subsequent routes leading to phytic acid are not fully clarified. The mechanism of a stepwise phosphorylation of Ins (*3*)P_1 to phytic acid was supported by the identification of specific inositol phosphate kinases (*6, 7*). More recently, recombinant inositol phosphate kinases cloned from rice (*OsIpk*) and barley (*HvIpk*) were shown to have multiple activities for a broad range of *myo*-inositol phosphate intermediates (*8*). In addition, free *myo*-inositol formed through dephosphorylation of Ins(3)P_1 is discussed as an intermediate in the biosynthesis of phytic acid. A *myo*-inositol kinase (MIK) has been isolated from maize, which phosphorylates *myo*-inositol but not Ins(3)P_1 (*9*).

Phytic acid is considered to be an antinutrient in food and feed. It limits the bioavailability of minerals such as iron, zinc, calcium, and selenium by formation of indigestible chelates (*10, 11*). Phytic acid is also poorly degraded in the digestive system of nonruminants (*12*). Therefore, animal feed producers and farmers must add phosphate to feed. Moreover, excreted phytic acid in manure is degraded by natural soil microorganisms, releasing phosphate, which contributes to eutrophication of water (*13*).

Owing to these disadvantageous effects, various efforts have been made to breed crop varieties low in phytic acid content. Mutation breeding has been successfully applied to generate low phytic acid (*lpa*) mutants of maize (*14–16*), barley (*17, 18*), rice (*19, 20*), soybean (*21–23*), and wheat (*24*). Three types of *lpa* mutations have been described in maize: The first reported

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lpa-1 mutants are characterized by a decrease in phytic acid content and a corresponding increase in inorganic phosphorus (*14*, *19*, *25*). In *lpa*-2 mutants the decrease in phytic acid content is accompanied by an accumulation of lower inositol phosphates (*14*, *18*). In *lpa*-3 mutants no lower inositol phosphates were detected, but an increase in *myo*-inositol content was described (*9*). Down-regulation of genes encoding enzymes considered to be essential for the biosynthesis of phytic acid, that is, MIPS or MIK, resulted in characteristic changes in the levels of certain crop metabolites (*9*, *22*, *26*).

The study objects of this investigation were two *lpa* rice mutants (*Os*-*lpa*-XS110-1 and *Os*-*lpa*-XS110-2) generated by γ-irradiation (*20*); the nature of the mutations underlying the decrease in phytic acid was not known. The aim was to compare these *lpa* rice mutants to the corresponding wild-type by means of metabolite profiling and to explore the usefulness of this approach to assist in the elucidation of the types of mutation resulting in the reduced content of phytic acid.

Metabolite profiling aspires to provide a comprehensive picture of the metabolites present in biological systems. It aims at extracting, detecting, identifying, and quantifying a broad spectrum of compounds in a single sample to provide a deeper insight into complex biological systems (*27*, *28*). The technique is being discussed as an additional tool for the safety assessment of genetically modified crops because of its potential to increase the probability to detect unintended effects (*29*, *30*). It is also considered as an approach extending and enhancing the power of functional genomics (*27*). Recently, a first attempt to apply metabolomic analysis to low phytic acid maize kernels has been reported (*31*).

MATERIALS AND METHODS

Chemicals. Phytic acid dodecasodium salt was obtained from Sigma-Aldrich (Taufkirchen, Germany). Internal standards (tetracosane, 5α-cholestan-3β-ol, phenyl-β-D-glucopyranoside, and *p*-chloro-L-phenylalanine), retention time standards (undecane, hexadecane, tetracosane, triacontane, and octatriacontane), phytic acid solution (technical 40% in water), and galactinol were purchased from Fluka (Taufkirchen, Germany). Reference compounds for quantification of *myo*-inositol, raffinose, and galactose were obtained from VWR International (Darmstadt, Germany). All other reagents and solvents were of analytical grade.

Rice Material. *Wild-Type Rice and lpa Mutants.* Low phytic acid mutant lines *Os*-*lpa*-XS110-1 and *Os*-*lpa*-XS110-2 were generated through γ-irradiation of the wild-type Xiushui 110 at the Irradiation Center of Zhejiang University, China. The selection of *lpa* mutants was based on screening of rice mutants by means of a colorimetric assay for high inorganic phosphorus (*32*). Low phytic acid mutants *Os*-*lpa*-XS110-1 and *Os*-*lpa*-XS110-2 developed from Xiushui 110 were classified due to their extent of increase in inorganic phosphorus compared to the wild-type (*20*).

Wild-type Xiushui 110 and *lpa* mutants *Os*-*lpa*-XS110-1 and *Os*-*lpa*-XS110-2 were grown in 2004 side by side in paddy fields at four locations in China (Jiaxing, Hainan, and two independent field trials in Hangzhou).

Sample Preparation. Rough rice grains were manually dehulled by means of a wooden rice dehuller and ground with a cyclone mill equipped with a 500 μm sieve (Cyclotec, Foss, Germany). The flour was freeze-dried for 48 h (Alpha 1-4 LSC, Christ, Germany) and stored at −18 °C.

Analysis of Inositol Phosphates. Isolation and separation of inositol phosphates were performed in accordance with previously described procedures (*33*, *34*). Half a gram of freeze-dried rice flour was defatted in 3 mL of petroleum benzene overnight. The defatted material was extracted under mechanical agitation with 10 mL of 2.4% hydrochloric acid for 2 h. After centrifugation (15 min, 25000*g*, 15 °C), 5 mL of the supernatant was diluted with 20 mL of deionized water. A silica-based quaternary ammonium anion exchanger column [Chromafix SB (M), 460 mg packing, Macherey-Nagel] connected to a vacuum manifold (Supelco) was conditioned with 5 mL of deionized water (1 mL/min). After application of the diluted sample extract (1 mL/min), the column was washed with 10 mL of deionized water and 10 mL of 5 mM hydrochloric acid (1 mL/min). Inositol phosphates were eluted with 5 mL of 2 M hydrochloric acid (1 mL/min) into a 10 mL graduated flask, which was filled with deionized water. Two milliliters of the purified extract was evaporated under vacuum to dryness by application of an ACTEVap evaporator (Advanced Chemtech). The residue was redissolved in 250 μL of HPLC mobile phase (485 mL of acetonitrile, 515 mL of 35 mM formic acid, and 10 mL of tetrabutylammonium hydroxide). The pH of the mobile phase was adjusted to 4.3 with 75% sulfuric acid. The sample was passed through a membrane filter (SPARTAN 13/0.45 RC, Whatman). Separation of inositol phosphates was performed on a macroporous polymer HPLC column PRP-1 (5 μm particle size, 150 × 4.6 mm; Hamilton) at a flow rate of 1 mL/min. The column was kept at 30 °C. The HPLC instrument (Kontron) was equipped with a refractive index detector ERC-7512 (ERMA CR).

Quantification of phytic acid ($InsP_6$) 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 ($InsP_5$, $InsP_4$, and $InsP_3$) were quantified on the basis of the calibration curve of phytate considering correction factors for lower inositol phosphates reported in the literature (*35*). Recoveries of $InsP_3$, $InsP_4$, $InsP_5$, and $InsP_6$ from rice spiked with the technical phytic acid solution (40% in water) were 101 ± 5, 108 ± 9, 108 ± 5, and 104 ± 2%, respectively (mean ± SD, $n = 5$). On the basis of a signal-to-noise ratio of 3:1, limits of detection were 0.13% ($InsP_3$), 0.11% ($InsP_4$), and 0.07% ($InsP_5$ and $InsP_6$), expressed as contents in freeze-dried rice.

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.

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.

Extraction Procedure. Six hundred milligrams of freeze-dried rice flour was weighed into a disposable cartridge (3 mL column volume, VWR International), which was sealed with plastic frits and connected to a vacuum manifold. The rice flour was soaked in 300 μL of methanol for 20 min. After removal of methanol by application of vacuum (25 mbar, 30 min) on top of the cartridge, lipids were eluted with 4 mL of dichloromethane within 20 min. Polar compounds were eluted with 10 mL of a mixture of methanol and deionized water (80 + 20, v/v) within 40 min.

Fractionation of Lipids. Fifty microliters of internal standard solution I and 150 μL of internal standard solution II were added to 4 mL of the lipid extract. After evaporation under vacuum to dryness by means of an ACTEVap evaporator (40 °C), 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 (50 °C).

A small amount of anhydrous sodium sulfate was placed on top of a 500 mg silica gel SPE cartridge (LiChrolut, VWR International) conditioned with 2.5 mL of *n*-hexane. Transmethylated lipids were redissolved in 250 μL of dichloromethane and transferred to the SPE cartridge. 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). After addition of 50 μL of retention time standard 1 to fraction II, both fractions were evaporated to dryness under vacuum (40 °C). The residue of fraction I was redissolved in 300 μL of dichloromethane, and 1 μL was injected into the gas chromatograph. After the flask had been flushed with argon, the residue of fraction II was redissolved in 250 μL of dry pyridine and silylated with 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide in an oil bath (15 min at 70 °C). One microliter of the sample was analyzed by gas chromatography.

Fractionation of Polar Extract. After the addition of internal standard solutions III (150 μL) and IV (150 μL), 1 mL of the polar extract was evaporated to dryness under vacuum (50 °C). The flask was flushed with argon, and the residue was redissolved in 300 μL of dry pyridine and silylated with 100 μL of trimethylsilylimidazole in an oil bath (15 min at 70 °C). The sample was diluted with 300 μL of *n*-hexane, and 300 μL of deionized water was added for selective hydrolysis of the silylated derivatives of organic acids and amino acids. After shaking and phase separation, 200 μL of the upper phase (fraction III) was mixed with 100 μL of retention time standard 1, and 1 μL was injected into the gas chromatograph.

To obtain fraction IV, 4 mL of the polar extract was evaporated to dryness under vacuum (50 °C). The residue was redissolved in 300 μL of a solution of hydroxyammonium chloride in pyridine (2 mg/mL). After 30 min of heating at 70 °C in an oil bath, 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was added while the flask was flushed with argon. After 15 min at 70 °C, 500 μL of *n*-hexane and 300 μL of deionized water were added to the silylated sample. After vortexing, the upper phase was discarded. Extraction with *n*-hexane was repeated two times. The aqueous phase was evaporated to dryness under vacuum (50 °C). The residue was redissolved in 200 μL of acetonitrile, and 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was added. The sample was allowed to stand for 60 min at 70 °C in an oil bath for silylation. It was cooled to room temperature and mixed with 50 μL of retention time standard 2, and 1 μL was analyzed by gas chromatography.

Quantification of Polar Compounds. Selected compounds of fraction III were quantified by triplicate analysis using phenyl-β-D-glucopyranoside as internal standard; phosphate was quantified using *p*-chloro-L-phenylalanine as internal standard. Recoveries determined by spiking 600 mg of freeze-dried rice flour with known amounts of reference standards [50 μL of aqueous solutions of galactose (0.4 mg/mL), *myo*-inositol (1.2 mg/mL), raffinose (8 mg/mL), and galactinol (0.5 mg/mL)] were as follows: galactose, 74%; *myo*-inositol, 58%; raffinose, 71%; galactinol, 69%.

Gas Chromatography. 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). The MS interface temperature was set to 320 °C and the 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.

Rice constituents were identified by comparing retention times with those of silylated reference compounds and by comparing mass spectra with the entries of the mass spectral library (NIST 2002).

Statistical Assessment. Rice samples were analyzed in triplicate. GC-FID data were acquired and integrated using Chrom-Card 2.3 (Thermo Electron). Peak heights (threshold = 1000 μV) and retention times were exported to Chrom*pare* version 1.1 for comparative assessment (*36*) (www.chrompare.com).

RESULTS

Analysis of Inositol Phosphates. Inositol phosphates isolated from the rice materials were analyzed by ion-pair HPLC. **Table 1** shows the data obtained for the wild-type Xiushui 110 and the *lpa* mutants *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 grown at four field trials in China. The phytic acid contents in the wild-type rice ranged from 0.7 to 1.1% and differed depending on the growing location. Lower inositol phosphates ($InsP_3$, $InsP_4$, and $InsP_5$) were not detected, except for the sample grown at Hainan (0.08% $InsP_5$). Compared to the wild-type, *lpa* mutants *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 exhibited significantly lower contents in phytic acid. The extents of reduction in phytic acid content were different for the two mutant lines and varied depending on the field trial. Overall, the reduction in phytic acid content in *Os-lpa*-XS110-1 (average = −46%) was more pronounced than that in *Os-lpa*-XS110-2 (average = −23%). Lower inositol phosphates ($InsP_3$, $InsP_4$, and $InsP_5$) were not detected in the mutant lines.

Table 1. Contents of Phytic Acid in Mutant Lines (*Os-lpa*-XS110-1 and *Os-lpa*-XS110-2) and Wild-Type Xiushui 110 Grown in Four Field Trials (Mean ± Standard Deviation, $n = 3$)

	phytic acid (%)			
	Hainan field trial	Jiaxing field trial	Hangzhou field trial 1	Hangzhou field trial 2
Xiushui 110	1.08 ± 0.06	0.73 ± 0.00	0.77 ± 0.05	1.06 ± 0.01
Os-lpa-XS110-1	0.49 ± 0.03	0.40 ± 0.00	0.51 ± 0.02	0.53 ± 0.03
Os-lpa-XS110-2	0.84 ± 0.03	0.51 ± 0.00	0.68 ± 0.01	0.78 ± 0.03

Metabolite Profiling. Metabolite profiling was performed according to a previously described extraction and fractionation scheme (*37*). Briefly, lipids and polar compounds were consecutively extracted from the freeze-dried rice flour. Lipids were transesterified in methanol and subsequently separated by solid phase extraction into a fraction containing fatty acid methyl esters (fraction I) and a fraction containing minor lipids, for example, sterols and free fatty acids (fraction II). Selective hydrolysis of silylated derivatives was applied to separate the polar extract into a fraction containing silylated sugars and sugar alcohols (fraction III) and a fraction containing organic acids and amino acids (fraction IV). The four fractions obtained were analyzed by gas chromatography (GC-FID). Fractions II and IV were silylated before GC analysis. Representative GC chromatograms of fraction III for wild-type rice and the two *lpa* mutants are shown in **Figure 1**. Peak heights and corresponding retention times were exported to Chrom*pare*, a software tool developed for comparative analysis of metabolite profiling data (*36*) (www.chrompare.com). Chrom*pare* automatically corrects retention time shifts on the basis of retention time standards and standardizes peak heights on the basis of internal standards added before the fractionation process. Comparison of metabolite profiles is performed by comparison of mean standardized peak heights based on triplicate analysis. Means are considered to be statistically significantly different if their confidence intervals ($p < 0.05$) are not overlapping. The magnitude of difference is calculated, and peaks observed in only one sample are reported as additional/missing peaks.

Results obtained by comparative metabolite profiling of wild-type Xiushui 110 and mutant line *Os-lpa*-XS110-1 grown at four locations are shown in **Table 2**. On average, a total of 116 peaks were included for comparison. Assessment via Chrom*pare* demonstrated that for each field trial approximately 40 of them, that is, 34%, were statistically significantly different ($p < 0.05$) between wild-type and *Os-lpa*-XS110-1.

However, only four of these peaks (three in fraction III and one in fraction IV) turned out to be statistically significantly different between the wild-type and *Os-lpa*-XS110-1 at all four field trials (i.e., consistent differences).

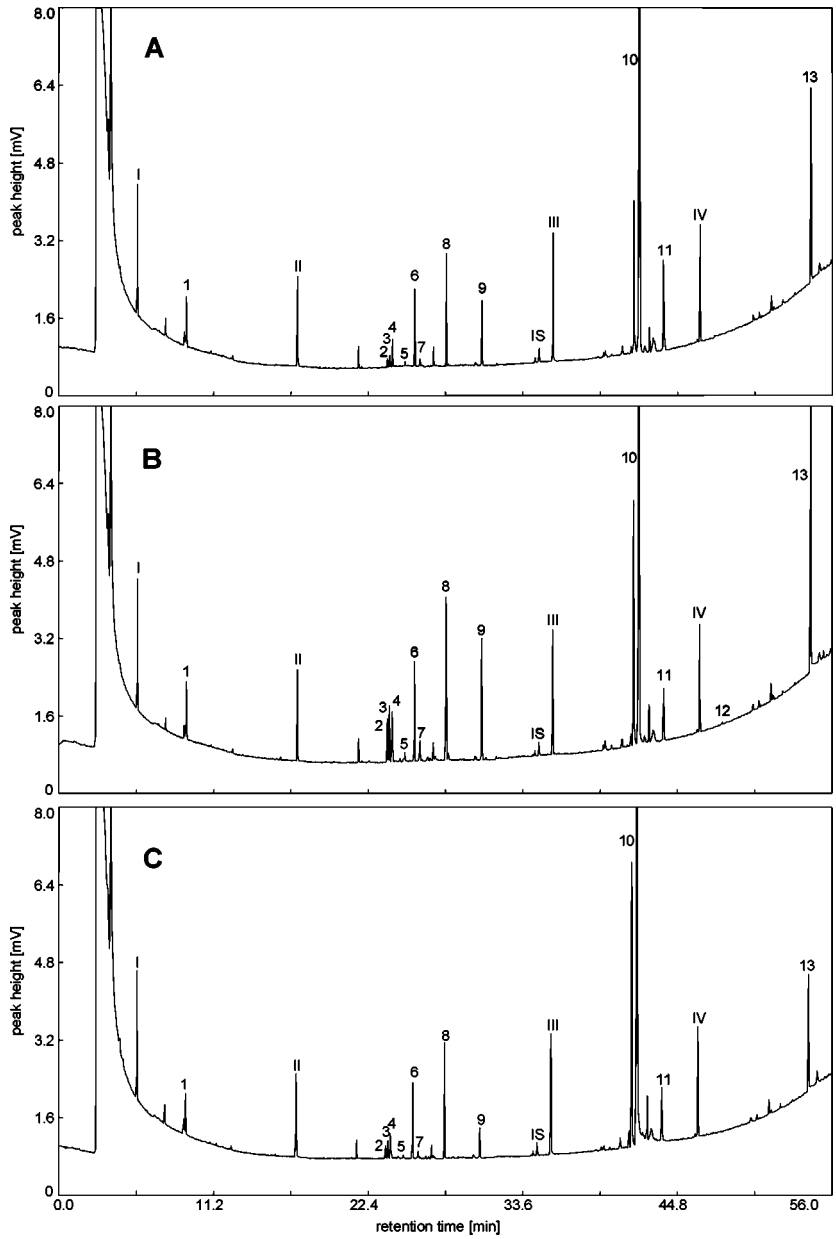

Figure 1. GC-FID chromatograms of fraction III obtained for the wild-type rice Xiushui 110 (**A**) and the two *lpa* mutants *Os-lpa*-XS110-1 (**B**) and *Os-lpa*-XS110-2 (**C**). Peaks 1–13: TMS derivatives of glycerol (1), fructose (2−4), galactose (5, 7), glucose (6, 8), *myo*-inositol (9), sucrose (10), trehalose (11), galactinol (12), and raffinose (13). Peaks I−IV: retention time standards undecane (I), hexadecane (II), tetracosane (III), and triacontane (IV). IS, internal standard phenyl-β-D-glucopyranoside.

Table 2. Peak-Based Comparison of Chromatograms Obtained by Metabolite Profiling of Wild-Type Xiushui 110 and Mutant Line *Os-lpa*-XS110-1 Grown in Four Field Trials

		field trial								
		Hainan		Jiaxing		Hangzhou 1		Hangzhou 2		
fraction	compound class	total[a]	diff[b]	total	diff	total	diff	total	diff	consistent differences[c]
I	FAME, hydrocarbons	30	10	27	1	26	8	15	1	
II	minor lipids	44	10	44	13	40	4	43	8	
III	sugars, sugar alcohols	11	3	11	5	10	6	12	5	3
IV	organic acids, amino acids	38	15	37	19	39	27	38	24	1
Σ I−IV		123	38	119	38	115	45	108	38	4

[a] Number of peaks included for comparison (peak height > 1000 μV). [b] Number of peaks statistically significant different between wild-type and mutant line ($p < 0.05$). [c] Number of peaks statistically significantly different between wild-type and mutant line at all four field trials.

The four compounds were identified by GC-MS as trimethylsilyl (TMS) derivatives of *myo*-inositol, galactose, and raffinose (fraction III) and TMS phosphate (fraction IV). Kovats retention indices, molecular masses, and mass spectral data of TMS derivatives of *myo*-inositol, galactose, and raffinose are shown in **Table 3**.

Table 3. Kovats Retention Indices (KI), Molecular Masses (*M*), and Major Fragment Ions of TMS Derivatives of Compounds Found To Be Consistently Significantly Different in Wild-Type Rice and *lpa* Mutants

peak[a]	compound	KI (DB-1)	*M*	major fragment ions [(*m*/*e*), rel intensity (%)]
9	*myo*-inositol	2152	612	305 (100), 217 (97), 318 (56), 147 (42), 73 (41), 191 (39), 306 (28), 319 (22), 204 (19), 218 (18)
13	raffinose	3541	1296	361 (100), 217 (53), 204 (41), 362 (30), 437 (24), 73 (19), 363 (15), 271 (13), 147 (13), 451 (12)
5	α-D-galactose	1906	540	204 (100), 191 (48), 217 (35), 73 (32), 147 (20), 205 (17), 206 (11), 75 (10), 207 (8), 192 (7)
7	β-D-galactose	1952	540	204 (100), 191 (53), 217 (48), 73 (35), 205 (23), 147 (21), 218 (10), 192 (9), 206 (8), 75 (6)
12	galactinol	3105	990	204 (100), 217 (32), 191 (20), 205 (19), 73 (16), 147 (10), 207 (9), 206 (9), 218 (8), 305 (7)
b	24-methylenecycloartanol	3472	512	207 (100), 379 (96), 422 (86), 407 (86), 95 (66), 73 (61), 135 (57), 107 (55), 147 (55), 69 (55)

[a] Numbers refer to chromatograms of fraction III shown in **Figure 1**. [b] Compound present in fraction II.

Table 4. Peak-Based Comparison of Chromatograms Obtained by Metabolite Profiling of Wild-Type Xiushui 110 and Mutant Line *Os-lpa*-XS110-2 Grown in Four Field Trials

		field trial								
		Hainan		Jiaxing		Hangzhou 1		Hangzhou 2		
fraction	compound class	total[a]	diff[b]	total	diff	total	diff	total	diff	consistent differences[c]
I	FAME, hydrocarbons	30	13	33	15	27	3	26	15	
II	minor lipids	47	8	41	30	43	9	38	27	1
III	sugars, sugar alcohols	10	5	10	1	10	2	11	3	1
IV	organic acids, amino acids	31	1	32	17	33	21	33	22	
Σ I - IV		118	27	116	63	113	35	108	67	2

[a] Number of peaks included for comparison (peak height > 1000 μV). [b] Number of peaks statistically significant different between wild-type and mutant line ($p < 0.05$). [c] Number of peaks statistically significantly different between wild-type and mutant line at all four field trials.

Results obtained by comparative metabolite profiling of the wild-type Xiushui 110 and mutant line *Os-lpa*-XS110-2 are shown in **Table 4**. Again, for each field trial on average 42% of the peaks included for comparison were statistically significantly different between wild-type and *Os-lpa*-XS110-2. However, consistent differences at all locations were observed for only two compounds that were identified as 24-methylenecycloartanol (fraction II) and *myo*-inositol (fraction III) (for mass spectral data see **Table 3**).

In a subsequent step the metabolites from fraction III, which had been shown to be consistently different between wild-type and mutants, were quantified. As shown in **Table 5**, the two *lpa* rice mutants exhibited characteristic quantitative patterns of these polar metabolites compared to the wild-type. The contents of *myo*-inositol, raffinose, and galactose were all statistically significantly increased in *Os-lpa*-XS110-1 compared to the wild-type. In contrast, *Os-lpa*-XS110-2 exhibited consistently decreased levels of *myo*-inositol compared to the wild-type. For raffinose, decreases in content in this mutant were also observed at all locations; however, for this compound the difference compared to the wild-type was statistically significant only in one field trial (Hainan).

Semiquantifications (comparisons of detector responses) were performed for 24-methylenecycloartanol and phosphate. As shown in **Figure 2A**, levels of phosphate were increased compared to the wild-type in both mutants at all field trials. The increase was much more pronounced in *Os-lpa*-XS110-1 (from +271 to +1300%) than in *Os-lpa*-XS110-2 (from +150% to +337%) (**Figure 2A**). On average, the levels of phosphate were 1.8 mg/g (wild-type), 14.1 mg/g (*Os-lpa*-XS110-1), and 5.5 mg/g (*Os-lpa*-XS110-2). Levels of 24-methylenecycloartanol were increased by up to 60% compared to the wild-type in both mutants, except for *Os-lpa*-XS110-1 grown at Jiaxing (**Figure 2B**).

Table 5. Contents of *myo*-Inositol, Raffinose, Galactose, and Galactinol in Wild-Type Xiushui 110 and Mutant Lines (*Os-lpa*-XS110-1 and *Os-lpa*-XS110-2) for Four Field Trials

	content[a] (μg/g of dry matter)			
	myo-inositol	raffinose	galactose	galactinol
Hainan				
Xiushui 110	47 ± 0	762 ± 24	17 ± 10	–[b]
Os-lpa-XS110-1	242 ± 53*	939 ± 5*	31 ± 4*	9 ± 1
Os-lpa-XS110-2	28 ± 2*	608 ± 32*	8 ± 1*	–
Jiaxing				
Xiushui 110	48 ± 6	446 ± 56	9 ± 2	–
Os-lpa-XS110-1	98 ± 4*	1227 ± 71*	27 ± 4*	4 ± 0
Os-lpa-XS110-2	26 ± 2*	342 ± 16	10 ± 20	–
Hangzhou 1				
Xiushui 110	40 ± 2	429 ± 44	10 ± 10	–
Os-lpa-XS110-1	104 ± 12*	1304 ± 61*	24 ± 2*	7 ± 1
Os-lpa-XS110-2	22 ± 1*	399 ± 29	7 ± 0	–
Hangzhou 2				
Xiushui 110	50 ± 3	227 ± 13	6 ± 1	–
Os-lpa-XS110-1	85 ± 1*	1131 ± 10*	14 ± 1*	6 ± 1
Os-lpa-XS110-2	26 ± 1*	192 ± 80	7 ± 0	–

[a] Mean ± standard deviation ($n = 3$). An asterisk indicates statistically significant difference compared to wild-type ($p < 0.05$). [b] Below the limit of quantification (4 μg/g).

DISCUSSION

The different phytic acid contents determined in the wild-type rice depending on the field trial confirm the environmental impact. This effect is in agreement with data reported for other conventional rice cultivars (*3*, *38*). Although the extent of decrease in phytic acid content in the mutants also differed depending on the field trial, the consistently less pronounced

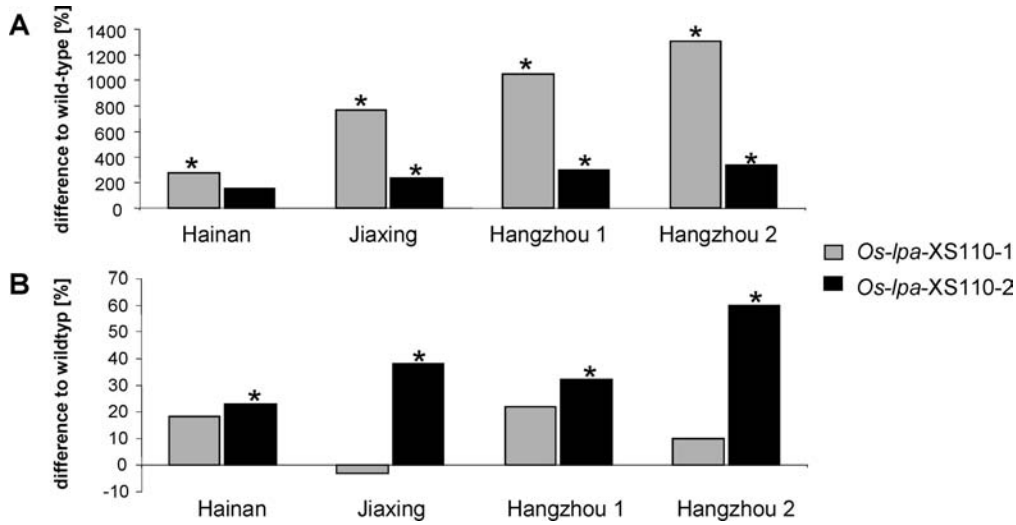

Figure 2. Differences in mean standardized peak heights (triplicate analysis) of phosphate (**A**) and 24-methylenecycloartanol (**B**) between wild-type Xiushui 110 and mutant lines (*Os-lpa*-XS110-1 and *Os-lpa*-XS110-2) grown in four field trials. An asterisk indicates a statistically significant difference from the wild-type ($p < 0.05$).

reduction in *Os-lpa*-XS110-2 was a first hint that different types of mutations might be involved.

The numbers of statistically significant differences ($p < 0.05$) in metabolites between wild-type rice and the mutants at each field trial (on average 34% for *Os-lpa*-XS110-1 and 42% for *Os-lpa*-XS110-2) are of the same order of magnitude as those determined for low phytic acid maize mutants. Application of a GC-MS metabolite profiling approach similar to the one employed in this study revealed 10% of the detected metabolites to be statistically significantly different ($p < 0.05$) between wild-type maize and an *lpa*-1 mutant and 29% to be different between wild-type maize and an *lpa*-3 mutant (*31*). It is also interesting to note that GC-MS metabolite profiling of potatoes revealed on average 50% of the polar metabolites analyzed to be statistically significantly different between tubers of transgenic lines altered in sucrose catabolism and wild-type controls grown in parallel in a greenhouse (*39*).

In the present study the assessment of the statistically significant differences observed between wild-type rice and *lpa* mutants for consistency revealed that the vast majority of differences are related to biological variability rather than to the mutation event and that only four (*Os-lpa*-XS110-1) and two (*Os-lpa*-XS110-2) metabolites remained as consistently different at all four locations. Comparable studies on transgenic rice demonstrated 22% of the metabolites analyzed to be statistically significantly different between insect-resistant Bt-rice and the isogenic control grown in the field under identical conditions; however, only five differences were found to be consistent when data sets from two field trials were compared (*40*).

The increase of phosphate content in both mutants in all field trials (**Figure 2**) is a change expected from the effect intended by the mutation. Other phosphorylated metabolites, which might be anticipated from the influence of the significant increase in phosphate on cell metabolism, were not detected. However, it has to be kept in mind that only low molecular weight compounds are covered by the used method. The increase in inorganic phosphate is typically used as a marker for the selection of *lpa* mutant lines (*14*, *18*, *19*). It has been shown that despite a block in phytic acid biosynthesis seed total phosphorus in *lpa* mutants is not decreased due to increased levels of inorganic phosphate (*41*).

The statistically significant increase in the content of 24-methylenecycloartanol consistently observed in *Os-lpa*-XS110-2 (**Figure 2**) cannot be explained. There is no obvious correlation to the biosynthesis of phytic acid.

On the other hand, *myo*-inositol, raffinose, and galactose are metabolites clearly linked to the biogenetic pathways leading to phytic acid. The first step in the biosynthesis of phytic acid is the conversion of D-glucose 6-phosphate to Ins(3)P_1 catalyzed by MIPS (*4*, *5*) followed by phosphorylation steps of *myo*-inositol monophosphate to phytic acid. In addition, free *myo*-inositol formed through dephosphorylation of Ins(3)P_1 by *myo*-inositol monophosphatase (MIP) acts as intermediate in the biosynthesis of phytic acid (*9*, *42*). As shown in **Figure 3**, the biosynthesis of raffinose is linked to the early biosynthetic pathway of phytic acid. Raffinose is synthesized by attaching galactose to sucrose involving *myo*-inositol as a carrier of galactose activated as galactinol (*43*, *44*).

***Os-lpa*-XS110-1 Mutant.** *Os-lpa*-XS110-1 exhibited statistically significantly increased levels of *myo*-inositol, raffinose, and galactose in all field trials. In addition, the content of the metabolically related sugar galactinol (which had not been covered by the Chrom*pare* assessment due to the thresholds set for comparisons) was shown to be increased compared to the wild-type (**Table 5**). Similar increases in raffinose and/or *myo*-inositol levels have been observed in *lpa*-2 barley (*45*) and *lpa*-2 maize mutants (*15*). However, in both cases the decrease in phytic acid content was accompanied by accumulation of lower inositol phosphates. In vitro activity assays indicated that an inositol phosphate kinase (Zmlpk) may be involved in the late steps of the phosphorylation of inositol phosphate. Accordingly, the mutation of the Zmlpk gene resulted in an accumulation of $InsP_3$, $InsP_4$, and $InsP_5$ (*15*). In the case of *Os-lpa*-XS110-1 no accumulation of $InsP_3$, $InsP_4$, or $InsP_5$ was detected.

Recently, an *lpa*-3 maize mutant was discovered exhibiting increased *myo*-inositol levels without accumulation of lower inositol phosphates (*9*). The gene affected by the mutation was shown to encode a *myo*-inositol kinase (MIK). This enzyme phosphorylates *myo*-inositol, but neither *myo*-inositol monophosphate nor $InsP_2$ is accepted as substrate.

The metabolite profiling data obtained for *Os-lpa*-XS110-1 indicate a disturbance in this latter biosynthetic pathway of phytic acid leading to a tailback of the metabolic flux. The

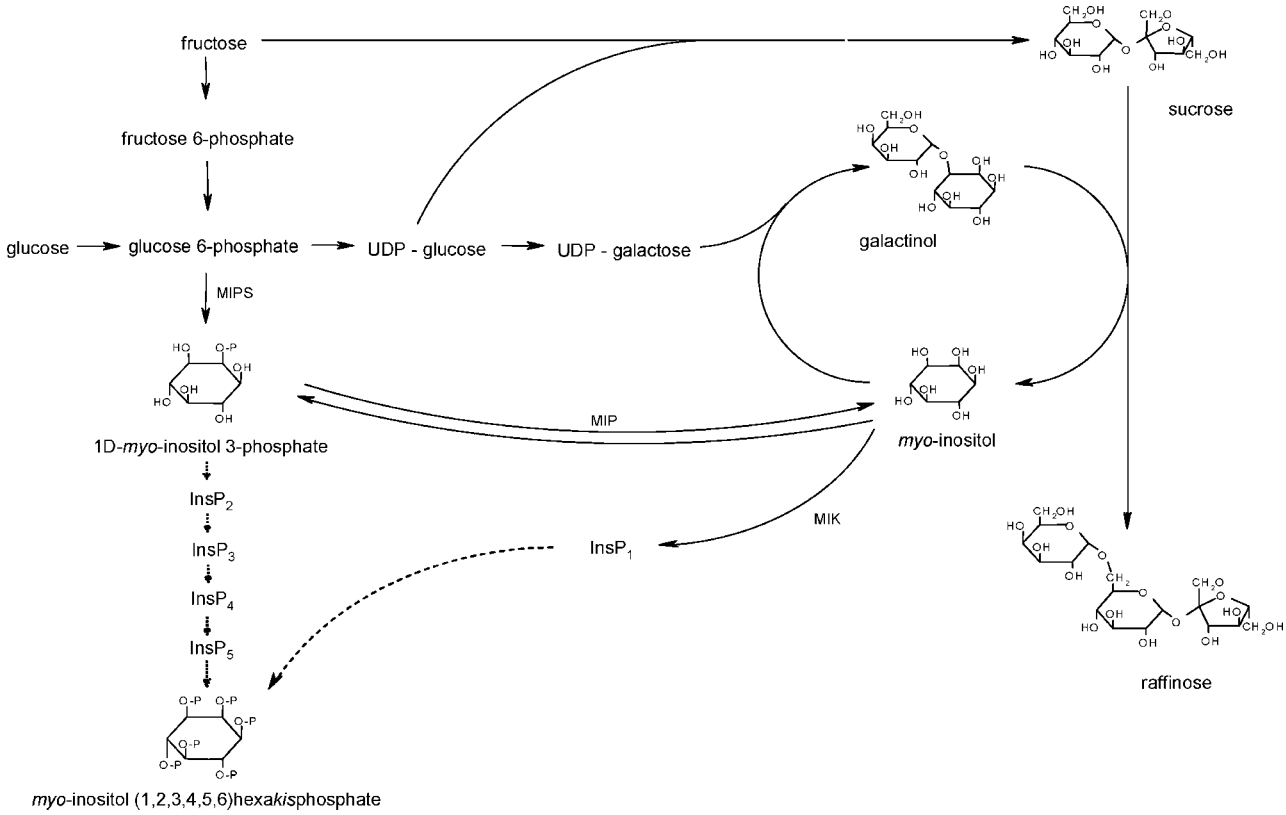

Figure 3. Plant biosynthetic pathways leading to phytic acid and raffinose. Dotted lines indicate not fully clarified pathways. MIPS, 1D-*myo*-inositol 3-phosphate synthase; MIP, *myo*-inositol monophosphatase; MIK, *myo*-inositol kinase.

increased contents of *myo*-inositol, raffinose, galactose, and galactinol in combination with the absence of $InsP_3$, $InsP_4$, and $InsP_5$ lend experimental evidence to the assumption that the mutation in *Os-lpa*-XS110-1 affected early phosphorylation steps of *myo*-inositol and may therefore be similar to the *lpa*-3 mutation in maize. This metabolite profiling-based prediction is consistent with mapping results, which placed the *lpa* mutation in *Os-lpa*-XS110-1 on a site very close to the locus that encodes the putative MIK gene in rice (*20*).

***Os-lpa*-XS110-2 Mutant.** The reduction of phytic acid content in *Os-lpa*-XS110-2 is accompanied by decreased levels of *myo*-inositol and raffinose without accumulation of lower inositol phosphates. Low phytic acid mutants of rice (*19*) and maize (*14*) that showed increased content of inorganic phosphorus but no accumulation of lower inositol phosphates have been classified as *lpa*-1 mutants. The phytic acid decrease in *lpa*-1 maize had originally been correlated to a mutation of the MIPS gene (*46*); however, it is now known that the *lpa1* mutation occurred in one of the multidrug resistance protein (MRP) genes, *ZmMRP3*, a gene encoding an ABC transporter (*47*). The *lpa1* mutation is located on a different chromosome from the MIPS gene in rice (*18*), but antisense inhibition of the MIPS gene also resulted in a significant increase in inorganic phosphorus (*48, 49*).

Mutation of the MIPS gene in *lpa* soybean caused reduced phytic acid level as well as reduced levels of *myo*-inositol and raffinose (*22*). In potato, inhibition of $Ins(3)P_1$ synthesis by antisense insertion of the MIPS gene resulted in decreased levels of *myo*-inositol, galactinol, and raffinose (*26*). Therefore, reduced levels of *myo*-inositol and raffinose in *Os-lpa*-XS110-2 may indicate a disturbance in this early part of the biosynthesis of phytic acid. When the absence of lower inositol phosphates is also taken into account, *Os-lpa*-XS110-2 may be similar to an *lpa*-1 maize mutant. Preliminary results of ongoing molecular genetic analyses are in agreement with this metabolite-based prediction. However, a final confirmation is still pending.

This study demonstrated the applicability of metabolite profiling for the detection of changes in the metabolite phenotype induced by mutation breeding. The combination of the described extraction and fractionation scheme with an efficient software enables the detection of statistically significant differences between wild-type and mutants. Investigation of materials grown at different locations allows consistent differences to be searched for, that is, to distinguish between natural variability and changes in metabolites induced by the mutation event. Metabolite profiling will not be able to replace classical biochemical studies on enzyme activities and genetic analyses for final confirmation of the mutation underlying a specific phenotype. However, it has the potential to assist in the elucidation of mutation events, and if used together with genetic tools, such as mapping, it would help to accelerate the discovery of the gene underlying the mutant phenotype. In addition, the screening of a broad spectrum of metabolites (from lipophilic to polar) increases the probability of detecting effects not intended by the mutation approach and thus may contribute to a safety assessment of mutants.

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