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ARTICLE

Changes in Protein Expression Profiles between a Low Phytic Acid Rice (*Oryza sativa* L. Ssp. *japonica*) Line and Its Parental Line: A Proteomic and Bioinformatic Approach

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The seed proteome of a low phytic acid (*lpa*) rice line (*Os-lpa*-XS110-1), developed as a novel food source, was compared to that of its parental line, Xiushui 110 (XS-110). Analysis by surfaced enhanced laser desorption ionization–time-of-flight mass spectrometry (SELDI-TOF MS) and two-dimensional gel electrophoresis (2-DE) allowed the detection of a potential low molecular weight biomarker and identification of 23 differentially expressed proteins that include stress-related proteins, storage proteins, and potential allergens. Bioinformatic analyses revealed that triose phosphate isomerase (TPI) and fructose bisphosphatealdolase (FBA), two major differentially expressed proteins, are involved in *myo*-inositol metabolism. Accumulation of globulin was also significantly decreased in the *lpa* line. This study demonstrates the potential of proteomic and bioinformatic profiling techniques for safety assessment of novel foods. Furthermore, these techniques provide powerful tools for studying functional genomics due to the possibility of identifying genes related to the mutated traits.

KEYWORDS: Novel food; unintended effects; proteomics; bioinformatics; SELDI; MALDI; 2-DE; *Oryza sativa*; phytic acid; mutagenesis; *myo*-inositol metabolism

INTRODUCTION

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate) is a phosphorylated derivative of *myo*-inositol and functions as the major storage form of phosphorus in plant seeds, where it typically represents from 65 to 80% of the mature seed total phosphorus content (*1*); in rice the phytic acid content of the seed can be up to 1.1% (*2*). Phytic acid can be viewed as a naturally occurring antinutrient in cereal grains and legumes due to its ability to form stable complexes with divalent cations such as Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ca^{2+} and proteins, therefore decreasing their bioavailability (*3*–*5*). Phytic acid is considered to be an important factor in iron deficiency that affects over 2 billion people worldwide and can cause problems such as impaired immune function and deleterious effects to both physical and mental development (*6*). Recent studies suggest that bioavailability of iron in vitro was significantly reduced by the presence of phytic acid in cell culture using Caco-2 cells as a model system (*7*). In addition, low bioavailability of zinc and iron from diets based on staple cereals such as rice and maize that are high in phytic acid is likely to be a major factor for deficiencies in these two micronutrients (*8*, *9*). In general, monogastrics, including humans, lack phytase activity and therefore are not capable of using phytic acid as a phosphorus source (*10*). In countries where rice forms a major part of the daily diet, it may thus be desirable to generate mutant varieties in which the seeds contain less phytic acid. To date, several low phytic acid (*lpa*) mutants of different food crops have been produced including barley (*11*), maize (*12*), soybean (*13*), and rice (*14*, *15*). However, the generation of novel foods by either conventional breeding techniques, including radiation/chemical mutagenesis, or genetic engineering can lead to unintended effects with possible consequences for food safety (*16*). Such unintended effects may be harmful or beneficial or, indeed, have no discernible consequences for either the safety profile of the crop or its agronomic vigor. To investigate whether these changes do have any consequences for food safety, it is therefore important to identify them using a range of profiling techniques together with associated bioinformatics, with a view to testing food safety. In the safety assessment of novel foods proteins are of special interest as they may be involved in the synthesis of toxins, antinutrients, or allergens (*17*–*19*). In addition, the screening methods developed may also give insight into the molecular mechanisms underlying the novel characteristics of the new food, and this understanding may be particularly

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useful in developing new strategies for selective breeding or mutagenesis.

In recent years low phytic acid crops have been developed and analyzed at both the transcriptome and metabolome levels (*20*, *21*). For example, using a metabolomic-based approach, Frank et al. (*21*) have shown that in the rice line *Os-lpa*-XS110-1 (the same material as used in the present study) the level of phytic acid was 46% lower compared to the parental line XS110, representing a significant reduction. Low phytic acid phenotypes are also usually identified on the basis of their high inorganic phosphorus (iP) content (*14*), which in many cases is assayed using the colorimetric method of Chen et al. (*22*). However, to our knowledge no previous studies have been carried out to investigate changes at the proteome level in *lpa* rice seeds, although proteomic approaches have been employed for comparative proteome analysis of different rice tissues under different growth stages and/or under biotic/abiotic stresses. Zang and Komatsu used a two-dimensional gel electrophoretic approach to identify osmotic stress-related proteins in rice leaves (*23*). Similarly, Hashimoto and Komatsu used 2-DE to identify differentially expressed proteins in rice root and leaves during cold stress (*24*). Interestingly, Liu et al. investigated the effect of temperature and caryopsis development on the rice seed proteome, demonstrating that elevated growth temperatures (35 °C) decrease the expression of allergen-like proteins (*2*).

The present study investigates the use of 2-DE and SELDI-TOF MS for food safety assessment and explores a number of bioinformatics techniques that can be used to analyze the resulting data. Furthermore, the study demonstrates that proteome analysis provides a powerful means of studying functional genomics, due to the possibility of identifying genes related to the mutated traits.

MATERIALS AND METHODS

Plant Material and Chemicals. The rice (*Oryza sativa* L. ssp. *japonica*) *lpa* line, *Os-lpa*-XS110-1, was derived from the commercial variety Xiushui 110, through mutagenesis by γ irradiation (*15*). *Os-lpa-XS110-1* and XS-110 rice were grown adjacent to one another under similar conditions in Hanghzou, China, and shipped to the United Kingdom as mature dried seeds.

All chemicals were from Sigma-Aldrich, unless otherwise indicated.

Preparation of Rice Proteins for 2-DE and SELDI Analyses. For the preparation of crude total soluble protein, 5 g of rice seeds was dehusked and the whole seed, including both the embryo and endosperm, were ground to a fine powder under liquid nitrogen using a mortar and pestle. The meal was then suspended in extraction buffer (10 mM Tris-HCl, pH 8, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA)) and mixed on an orbital shaker at 4 °C for 2 h before being centrifuged at 13000*g* for 15 min. Total soluble protein content of the extract was quantified by the Bradford assay (*25*). Samples (containing 450 μg of total protein) for 2-DE separation were desalted using GE Healthcare's 2-D cleanup kit following the manufacturer's instructions before being subjected to isoelectric focusing (IEF). All analyses were carried out on batches of seed grown concurrently in adjacent fields in China.

Protein Profiling: SELDI-TOF-MS (SELDI). Weak cation exchange (CM10) array chemistry ProteinChips (CiphergenBiosystems, Inc.) were employed for protein profiling. Protein samples from *Os-lpa*-XS110-1 or XS-110 were diluted 1:1 with 6 M guanidine hydrochloride in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.5, followed by a 1:40 dilution with 100 mM Tris-HCl, pH 7.5 (binding buffer); CM10 chips were pre-equilibrated three times with 150 mL of binding buffer on a platform shaker (250 rpm for 5 min); 5 mL of each diluted sample was applied to the arrays and incubated for 16 h at 22 °C; the arrays were washed three times with 100 mM Tris-HCl, pH 4 (platform shaker, 250 rpm for 5 min), followed by three rinses with deionized water, and air-dried. Half a milliliter of a saturated solution of the sinapinic acid matrix in 50% (v/v) acetonitrile (ACN) and 0.5% (v/v) trifluoroacetic acid (TFA) was applied. The arrays were analyzed using a Protein Chip System Series 4000 (CiphergenBiosystems, Inc.) mode: 25 kV positive ions; focus mass, 6000 Da; matrix attenuation, 1000 Da; sampling rate, 400 MHz; data shots, 10; energy, 2000 nJ). Mass accuracy was calibrated externally using the all-in-one peptide molecular mass standard (CiphergenBiosystems, Inc.). Peaks between 2000−10000 Da were detected using Ciphergen Express software (CiphergenBiosystems, Inc.). Under the selected mass range, each spectrum detected, on average, 34 peaks.

Protein Profiling: Two-Dimensional Gel Electrophoresis (2-DE). 2-DE was carried out in triplicate on the six samples (three parental and three *lpa*). Briefly, 450 μg of the protein extracts was diluted in 340 mL of rehydration buffer (7 M urea; 2 M thiourea; 0.75% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS); 0.75% (v/v) Triton X-100; 100 mM 1,4-dithiothreitol (DTT); 2% (v/v) immobilized pH gradient (IPG) buffer pH 3−10 (GE Healthcare)), containing a trace of bromophenol blue, loaded onto 18 cm immobiline DryStrip gels pH 3−10 (GE Healthcare), and run on an EttanIPGphor II (GE Healthcare; rehydration, 20 h at 20 °C; 500 V for 1 h; 500−1000 V in 1 h; 1000−10000 V in 3 h; 10000 V for 55 min). IPG strips were then equilibrated for 20 min in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (w/v) glycerol, 2.3% (w/v) sodium dodecyl sulfate (SDS), and 1% (w/v) DTT, followed by 20 min in the same solution but with DTT replaced with 4% (w/v) iodoacetamide. The IPG strips were then applied to 20 × 26 cm, 1.5 mm thick, 12.5% acrylamide gels and were run in an EttanDALTsix gel unit (GE Healthcare) for 20 h and 1.5 W per gel at 10 °C. The running buffer was 25 mM Tris base, 192 mM glycine, and 0.1% SDS. Finally, the gels were stained with Coomassie Brilliant Blue (CBB) (0.1% solution of Phastgel-blue R-350; GE Healthcare, in 40% methanol, 7% acetic in deionized H_2O) overnight and destained in 40% methanol and 7% acetic acid at room temperature on an orbital shaker. The stained gels were scanned on a calibrated Ettan Gel Imager (GE Healthcare) before being subjected to image analysis.

Image Analysis. ProgenesisSameSpots software (Nonlinear Dynamics) was used for gel analysis. The spots were matched between all of the gels using the SameSpots approach. Background subtraction and volume normalization were carried out. Artifacts and nonprotein spots were removed by editing the template image and were expanded to all gels using the SameSpots option. Gels were also subjected to statistical analysis (*t* test). Protein spots of interest were excised using Harris spot cutters (Sigma) before being subjected to in-gel digestion with trypsin and matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF MS) or MS/MS analysis for subsequent identification.

In-Gel Trypsin Digest and MS. Excised spots were washed with 25% (v/v) methanol and 7% (v/v) acetic acid for 12 h at room temperature and destained with 50 mM NH_4HCO_3 in 50% (v/v) methanol for 1 h at 40 °C. The gel pieces were then incubated in 10 mM DTT and 100 mM NH_4HCO_3 for 1 h at 60 °C; followed by 40 mM iodoacetamidem and 100 mM NH_4HCO_3 for 30 min at room temperature. The gel pieces were minced, dried, rehydrated in 100 mM NH_4HCO_3 containing 1 pmol of trypsin, and incubated at 37 °C overnight. The digested peptides were extracted from the gel slices with 0.1% TFA in 50% (v/v) ACN/water. The peptide solution was dried, resuspended in 30 μL of 0.1% TFA in 5% ACN, and desalted on Zip Tip C18 pipet tips (Eppendorf). The peptide solution was mixed with the matrix solution (50% ACN solution saturated with α-cyano-4-hydroxycinnamic acid (HCCA), and applied to a MALDI plate. MALDI MS was performed using ABI Voyager-DE STR Biospectometery (Applied Biosystems, Framingham, MA), and an LTQ FT Ultra Hybrid mass spectrometer (Thermo Electron Corp.) was used for MS/MS analysis. Proteins were identified by analyzing their peptide fingerprints or MS/MS traces using a Mascot search engine (Matrix Science).

Bioinformatics Analysis of Identified Proteins. Identified proteins were annotated using two different ontologies, and the resulting records were examined for over-representation of terms. (i) *Gene Ontology* (*GO*) *annotation:* Protein records were annotated with GO terms using the GOFigure tool (*26*). This resulted in one or more annotations being applied to 19 of the 23 proteins that were differentially expressed. (ii) *Kyoto Encyclopedia of Genes and Genomes* (*KEGG*) *annotation:* Protein records were annotated with KEGG Orthology (KO) terms using the KOBAS program (*27*). This resulted in one or more annotations being applied to 14 of the 23 proteins.

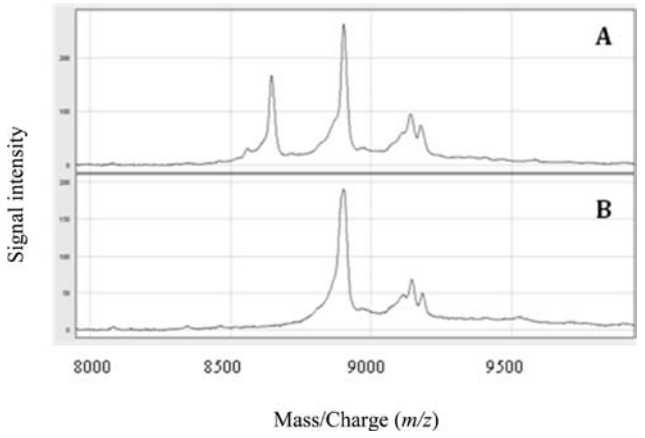

Figure 1. SELDI-TOF MS comparative proteome profiling of *Os-lpa*-XS110-1 (**A**) and XS-110 (**B**). The soluble fraction of the extracts was subjected to analysis using weak cation exchange array (CM10). A protein with *m/z* of 8642 Da was consistently detected in *Os-lpa*-XS110-1 but not XS-110. This relatively small protein might well be undetectable using 2DE.

Custom annotation files were written for the *Oryza sativa* proteome, linking UniProt IDs with GO and KO terms. A custom ontology file was also written to display (KEGG) pathways in a directed acyclic graph (DAG), using the KO for *O. sativa* as a starting point. Networks were drawn in Cytoscape 2.4 (*28*) using the BiNGO plugin (*29*). BiNGO uses the hypergeometric distribution with Bonferroni multiple-testing correction to assess which terms of a given set are over-represented in a list of proteins of interest, in comparison to the set of terms for that organism's proteome as a whole.

A network of statistically over-represented GO terms was drawn using BiNGO, with a *p* value cutoff of 0.05. BiNGO was also used to draw a network of KEGG orthology terms represented in the list of proteins, by applying no *p* value cutoff to the data collected.

RESULTS AND DISCUSSION

Biomarker Detection by SELDI Analysis. Protein extracts from seeds of rice lines *Os-lpa*-XS110-1 (low phytic acid) and XS-110 (parental) were analyzed using CM10 arrays. Protein profiles revealed the presence of an 8642 Da protein, which appeared to be unique to the Os-*lpa*-XS110-1 line (**Figure 1**). This result was consistently obtained from several independent extractions; however, subsequent attempts to identify the protein were unsuccessful.

The method developed here for the preparation of protein extracts from rice seeds, followed by SELDI analysis, provides a rapid method for distinguishing between *Os-lpa*-XS110-1 rice used in this study and XS-110. This approach could be adapted for other modified crops and therefore provide a rapid screening technique, once suitable biomarkers have been determined. SELDI-TOF MS has also been used to characterize recombinant protein expression in genetically modified (GM) crops (*30*).

2-DE Analysis of *Os-lpa*-XS110-1 and XS-110 Lines. 2-DE resolved 800 discrete protein spots in gels derived from *Os-lpa*-XS-110, whereas those from XS110-1 contained 825 discrete protein spots (**Figure 2**). These values are in keeping with the number of protein spots previously reported for rice seed (*31*).

Availability of the fully annotated rice genome (http://rice.plantbiology.msu.edu/) enabled identification of differentially expressed proteins between the two lines to be made. In total, 23 proteins that were differentially expressed (with a >2-fold change) in *Os-lpa*-XS110-1, when compared to XS-110, were identified (**Table 1**). Interestingly, these 23 proteins were represented by 39 protein spots in the 2-DE gel data sets, thus suggesting that *Os-lpa*-XS110-1 is different not only in protein expression from XS-110 but also in secondary modifications of some of these proteins (**Table 1**).

In the present study differential expression not only resulted in a change in the level of expression of specific proteins but, for others resulted in a complete absence of the gene product. Sixteen protein spots present in XS-110 were not detected in the *Os-lpa*-XS110-1 line, of which 12 were successfully identified as α-amalylase/subtilisin inhibitor, cytosolic 6-phosphogluconate dehydrogenase, putative dnaK-molecular chaperone Bip, endosperm lumenal binding protein, formate dehydrogenase (mitochondrial), putative globulin, 19 kDa globulin precursor, lactoylglutathionelysase, nascent polypeptide associated complex α-chain, pyruvate decarboxylase isozyme 3, TPI, plus one hypothetical protein (**Table 1**). There were also three protein spots (two proteins) that were detected only in *Os-lpa*-XS110-1, including proxiredoxin, and two protein spots both representing a small GTP-binding protein (**Table 1** and **Figure 2**).

In addition to proteins that were unique to either *Os-lpa*-XS110-1 or XS-110, the former contained 12 protein spots (representing 8 different proteins) that were decreased and 11 that were increased in their expression levels (**Figures 2** and **3**). Proteins with decreased expression levels in the *Os-lpa-XS110-1* line included α-amylase/subtilisin inhbitor, dnaK-type chaperone, globulin, a 19 kDA globulin precursor, glyoxalase, pyruvate decarboxylase, nascent polypeptide complex, and TPI (**Table 1**). Conversely, those with increased expression included 3-*β*-hydroxysteroid dehydrogenase/isomerase, dnaK-type molecular chaperone, FBA (also called aldolase C1), glyceraldehyde-3-phosphate dehydrogenase (GPDH), heat shock protein, hypothetical protein, ketol acid reductoisomerase, enolase, myosin heavy chain, serine proteinase inhibitor (serpin), and OSJNBb118P14.11 protein (**Table 1**). However, some of the identified proteins are products of multigene families, and expression of one gene product could be independent from expression of similar proteins by other genes (**Table 2**).

Analysis of Protein Annotation. *KEGG Orthology Terms.* FBA, which is responsible for converting *β*-D-fructose-1,6P2 to glyceraldehyde-3-phosphate and D-fructose-1-phosphate to glycerone-phosphate and D-glyceraldehyde, was up-regulated in *Os-lpa*-XS110-1, whereas TPI, which catalyzes dihydroxyacetone phosphate to GAP (reversible), was down-regulated in this low phytic acid line. The up- and down-regulation of these two enzymes is likely to lead to an increase in glyceraldehyde-3-phosphate, via fructose mannose metabolism, supplying the glycolysis/gluconeogensis pathways (**Figure 4**). The up-regulation of GPDH (NADP+) (EC 1.2.1.12 = EC 1.2.1.9 (+NADP)) could also lead to the channeling of this excess glyceraldehyde-3-phosphate into gluconeogenesis (**Figure 4**).

Over-represented GO Terms. The overall impressions given by the over-represented GO (gene ontology) terms is to reinforce those observed in the KEGG terms that are prevalent. FBA activity is represented, as is GPDH (NADP+) activity. In the "biological process" portion of the ontology, there appears to be a general trend that metabolism and catabolism have been affected, particularly with respect to sugars.

Presence of Allergenic Proteins in Low Phytic Acid Rice. Almost all plant food allergens are storage or defense-related proteins enabling the plant to resist biotic or abiotic stress (*32*). Allergenicity of food crop proteins are determined by factors such as their membership of a certain protein family, their abundance, their stability to processing and digestion, and their sequence homology to known allergens (*33*). Producing novel food by techniques such as *γ* irradiation may alter the incidence of allergens (*34*). Furthermore, in terms of food safety, it has been suggested that proteins introduced into biotechnology-derived crops should not show amino acid sequence similarity to known

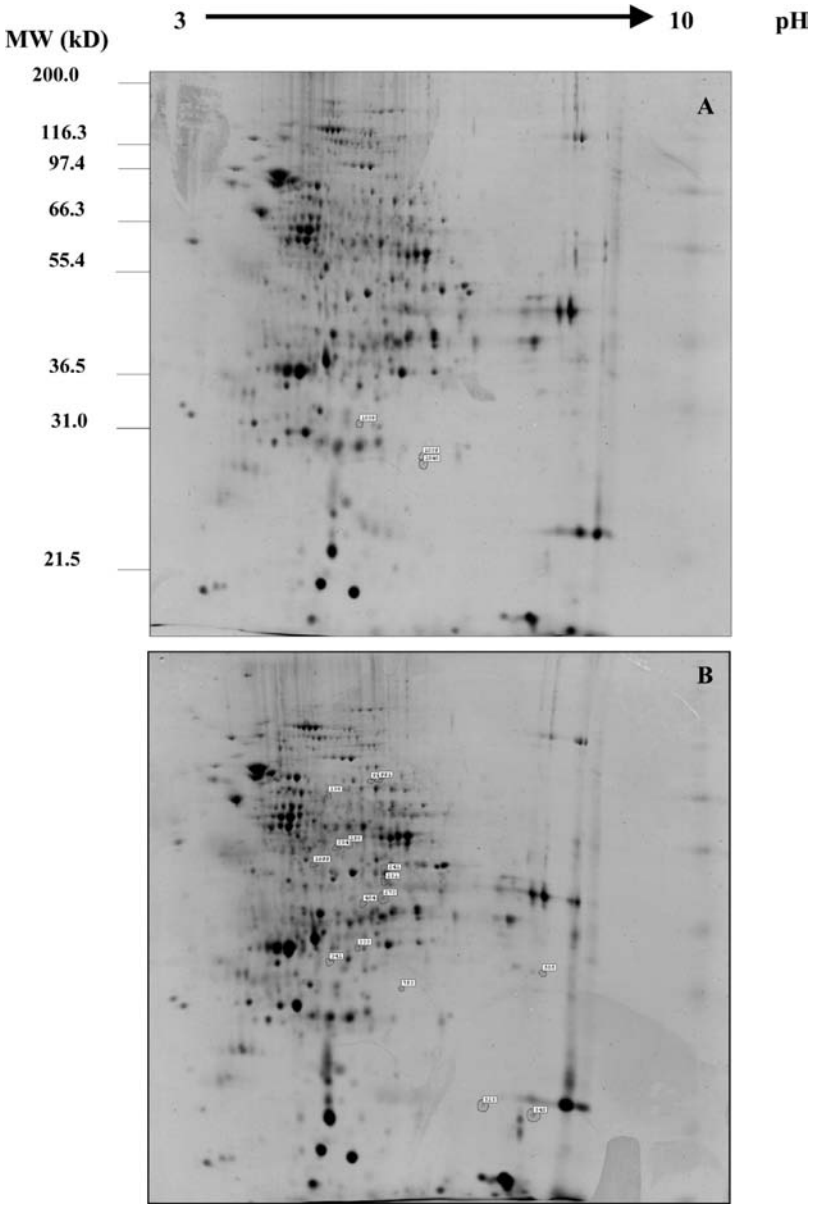

Figure 2. 2-DE maps of *Os-lpa*-XS110-1 and XS-110 seed proteins. Proteins (450 μg) were extracted from seeds and desalted as described under Material and Methods. IEF was carried out in the pH range of 3−10 on 18 cm IPG strips in the first dimension, and SDS-PAGE was performed using 12.5% acrylamide gel in the second dimension. Proteins were detected by CBB staining. The presence or absence of the proteins was detected by ProgenesisSameSpots software. Panel **A** shows *Os-lpa*-XS110-1 reference gel image, and panel **B**, XS-110. Proteins that are detected in *Os-lpa*-XS110-1 only and are absent in XS-110 or vice versa are highlighted with boxes that also show their relative distribution in the gel.

allergens or pharmacologically active proteins (*35*). It is thought that proteomics can help to identify allergenic proteins present in plants (*36*, *37*). The first reported allergens in rice were 14−16 kDa proteins, which were detected using sera from patients allergic to rice (*38*). A 16 kDa protein was later recognized as a major rice allergen. This protein has significant amino acid homology to barley trypsin inhibitor and wheat α-amylase inhibitor (*39*).

Using two-dimensional immunoblotting and mass spectrometry, Sander et al. (*40*) investigated the most frequently recognized allergens in wheat flour. In their study, more than 100 IgE binding spots were identified from which 2 were homologous to barley GAPDH, 1 to barley TPI, and a homologous protein to wheat serine proteinase inhibitor. In the present study some of the differentially expressed proteins in *Os-lpa*-XS110-1 or XS-110 were identified as potential allergens, as discussed below (**Table 3**).

(*a*) *Glyoxalase I* (*Lactoylglutathione Lyase*). Usui et al. characterized a 33 kDa allergenic protein from rice and identified it as the enzyme glyoxalase I (*41*). Glyoxalase contains one zinc atom that is essential for its activity (*42*). Interestingly, *Os-lpa*-XS110-1 has a lower Zn content than the parental line XS-110 (*43*), and this could account for the altered glyoxalase accumulation in the seeds of this low phytic acid mutant.

(*b*) *Globulin Proteins*. In some food crops such as peanuts, soybean, lentils, and sesame, globulins have been found to be allergenic (*32*, *44*, *45*). These proteins belong to the cupin superfamily of food allergens that comprise the major globulin storage proteins from legumes (*33*) . Of potential nutritional significance was the finding that the globulin content in *Os-lpa*-XS110-1 was significantly lower than in XS-110 (**Table 1**). Association of low phytic acid and low α-globulin accumulation in *Os-lpa*-XS110-1 might well be due to the binding properties of α-globulin to phytic acid (*46*).

Table 1. Proteome Analysis of Parental (XS-110) and Low Phytic Acid (*Os-lpa*-XS110-1) Rice Seeds following 2-DE and MALDI-TOF Analyses[a]

A. Proteins Decreased or Absent in *Os-lpa*-XS110-1

spot	protein name	gene name	UniProt Accession code	theor p*I*	obsd p*I*	theor M_r (kDa)	obsd M_r (kDa)	fold change	sequence coverage %/ Mascot score
WT-545[b]	α-amylase/subtilisin inhibitor	RASI	IAAS_ORYSJ	8.6	8.8	21.4	21.0	na	37.122
WT-525[b]					8.2		22.0	na	43.074
WT-776					9.1		21.2	3.35	46.220
WT-785					8.0		16.3	3.06	46.104
WT-991	pullulanase	OSJNBa0019G23.2	Q7X834_ORYSJ	5.8	6.2	106.5	85.0	na	28.142
WT-990					6.4		85.0		20.102
WT-204	cytosolic 6-phosphogluconate dehydrogenase	Os06g0111500	Q7FRX8_ORYSA	5.8	5.9	52.7	62.0	na	61.249
WT-505	putative dnaK-type molecular	P0036E06.29	Q6Z7B0_ORYSJ	5.1	9.0	73.4	33.0	na	18.130
WT-138	chaperone BiP				5.3		75.0	na	24.087
WT-763					6.9		31.0	na	18.135
WT-236					7.5		64.4	2.93	14.442
WT-198	endosperm lumenal binding protein	Bip	O24182_ORYSA	5.3	6.1	73.5	65.0	na	14.055
WT-251	formatedehydrogenase,	Os06g0486800,	FDH1_ORYSJ	6.6	6.6	41.3	50.0	na	38.072
WT-241	mitochondrial	LOC_Os06g29180			6.6		53.0		24.079
WT-273	putative globulin	OSJNBb0060J21.10	Q852L2_ORYSJ	6.4	7.0	52.1	48.0	na	37.105
WT-341					5.9	51.0	36.0	na	29.086
WT-351					5.2	51.1	36.1	6.20	29.115
WT-382[b]	19 kDa globulin precursor	Os05g0499100,	GL19_ORYSJ	7.5	5.6	21.1	22.3	2.10	21.225
WT-388[b]		LOC_Os05g41970			6.8		23.6 18.8	2.30	24.199
WT-348	lactoylglutathione lyase (glyoxalase I)	GLX-I	LGUL_ORYSJ	5.5	5.8	32.6	36.7	3.90	07.016
WT-378	nascent polypeptide associated complex α chain	P0504E02.33	Q8RUI4_ORYSJ	4.3	4.2	22.1	22.8	2.50	43.129
WT-133	pyruvate decarboxylase isozyme 3	PDC3	PDC3_ORYSA	5.9	5.5	62.6	80.2	3.50	37.109
WT-350	triosephosphate	TPI	TPIS_ORYSJ	5.3	5.1	27.1	36.1	5.10	84.121
WT-359	isomerase (TPI)				4.6		32.0	3.23	84.145

B. Proteins Increased in *Os-lpa*-XS110-1

spot	protein name	gene name	UniProt Accession code	theor p*I*	obsd p*I*	theor M_r (kDa)	obsd M_r (kDa)	fold change	sequence coverage %/ Mascot score
lpa-1038	1-Cys peroxiredoxin A	Os07g0638300, LOC_Os07g44430	REHYA_ORYSA	5.9	5.9	24.0	31.5	na	19.037
lpa-1039[b]	small GTP-binding protein (Ran2)	OsRan2	Q7GD79_ORYSJ	6.6	6.7	25.0	28.0	na	33.061
lpa-1040[b]					6.9		26.0	na	13.129
lpa-086[b]	methionine synthase	N/A	Q8W529_MAIZE	5.7	5.8	84.5	98.0	2.08	31.88
lpa-173	enolase	ENO1	ENO_ORYSJ	5.7	5.5	48.0	48.0	2.18	18.053
lpa-244	fructose biphosphate	N/A	Q42476_ORYSA	8.4	7.6	38.8	48.2	11.60	23.394
lpa-937	aldolase; FBA (aldolase C-1)				7.2		37.1	8.60	07.099
lpa-258					8.8		51.4	3.20	52.116
lpa-1095	glyceraldehyde-3-phosphate dehydrogenase	P0528B09.18	Q6Z9G0_ORYSJ	6.5	6.7	53.4	71.5	3.70	43.091
lpa-1101	heat shock protein 70	N/A	Q39641_CUCSA	5.1	4.3	75.4	94.1	3.00	22.110
lpa-960					5.0		75.0	2.00	22.096
lpa-346	putative 3-β hydroxysteroid dehyrogenase/isomerase	OSJNBb0081B07.22	Q94Hj5_ORYSJ	6.3	6.2	27.9	33.2	3.92	03.106
lpa-380	hypothetical protein	OJ1008_D08.3	Q6L578_ORYSJ	10.3	4.0	15.9	22.8	2.70	54.067
lpa-337	hypothetical protein	OSJNBb0118P14.11	QF7XP20_ORYSJ	4.9	4.6	33.8	38.4	2.47	16.039
lpa-257	putative serpin (serine proteinase inhibitor)	OSJNBb0007E22.7	Q75H81_ORYSJ	5.7	5.9	42.1	51.4	3.40	69.311

[a] *lpa* spot picked from *lpa* gels. WT, spot picked from XS-110 gels. na, not applicable; *P* values for all identifications were <0.05. [b] Identification confirmed by MS/MS.

(*c*) *Bifunctional* α-*Amylase*/*Subtilisin Inhibitors*. In general, proteinase inhibitors show allergenic properties (*47*). As such inhibitors are thought to play a role in plant defense against pathogens and/or insect pests, the reduced levels of expression of enzyme inhibitors of this type and elevated level of a serpin in *Os-lpa*-XS110-1 (**Table 3**) may alter the

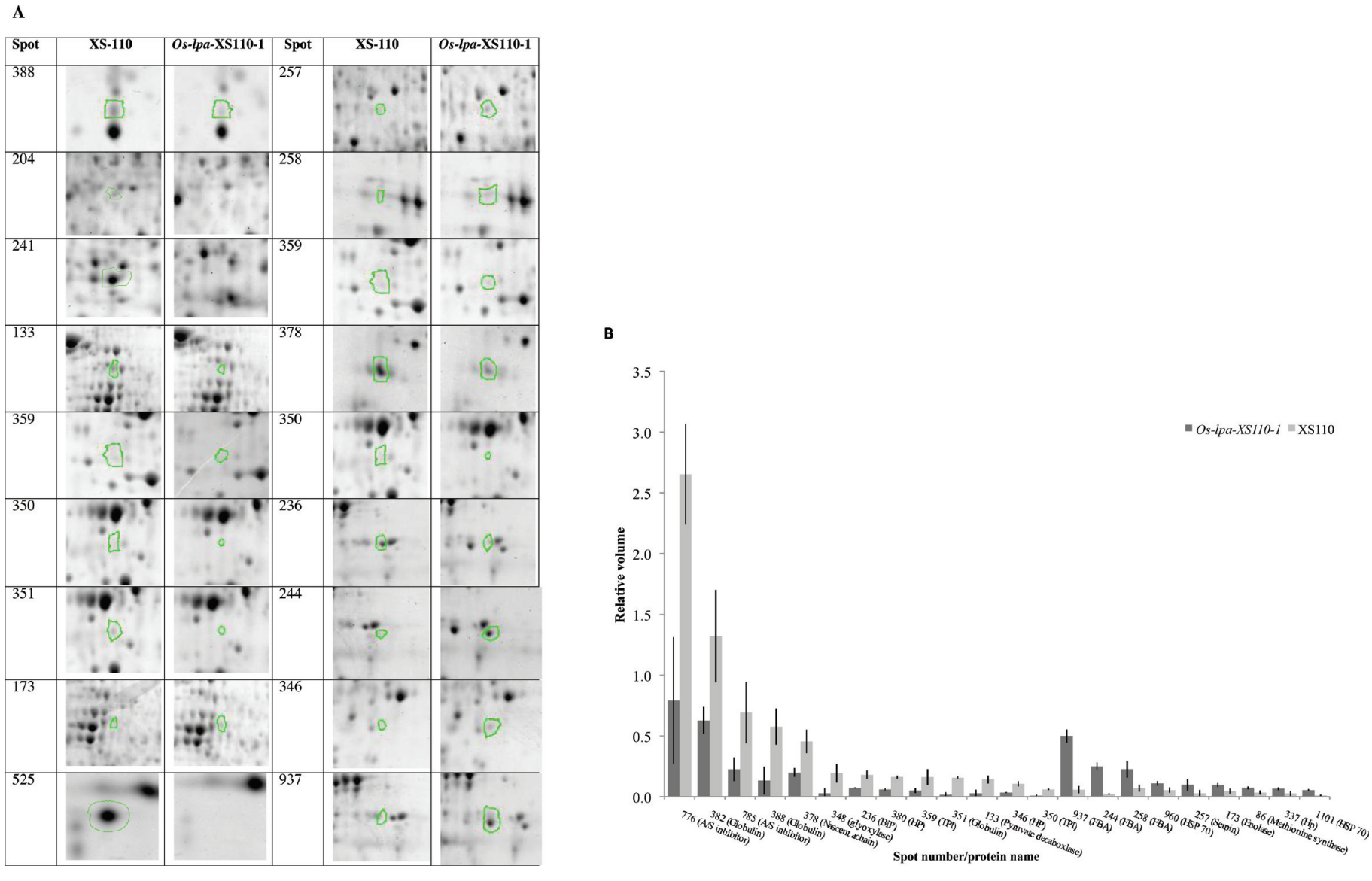

Figure 3. Representative examples of differentially expressed seed proteins between Os-*lpa*-XS110-1 and XS-110 rice lines and their relative abundance: (**A**) separate 2-D gel images of differentially expressed proteins; (**B**) quantitative changes of the up- or down-regulated proteins in *Os-lpa*-XS110-1 and XS-110. Data indicate relative volume of the spots that was calculated by ProgenesisSameSpots software. The *X*-axis denotes the protein spot number (**Table 1**), and the *Y*-axis shows relative levels of protein accumulation. The bars in the graphs represent an average volume ± SD of three gel replicates. A/S, α-amylase/subtilisin inhibitor; HP, hypothetical protein; HSP, heat-shock protein.

Table 2. Examples of Multigene Families Related to the Identified Proteins in This Study[a]

gene	total	**no. of genes**/location
dnaK-type molecular chaperon	9	**2**/Chr 1, **1**/Chr 12, **1**/Chr 2, **1**/Chr 3, **2**/Chr 5, **2**/Chr 6
formate dehydrogenase	2	**2**/Chr 6
fructose bisphosphate aldolase	8	**2**/Chr 1*, **1**/Chr 10, **1**/Chr 11,[b] **1**/Chr 12, **1**/Chr 5, **1**/Chr 6, **1**/Chr 8
globulin	5	**4**/Chr 5, **1**/Chr 9
GTP binding protein	54	**12**/Chr 1, **1**/Chr 10, **3**/Chr 11, **2**/Chr 12, **11**/Chr 2, **6**/Chr 3, **1**/Chr 4, **7**/Chr 5, **4**/Chr 6, **4**/Chr 7, **3**/Chr 8
peroxiredoxin	5	**1**/Chr 1, **1**/Chr 2, **1**/Chr 4, **2**/Chr 7
serine proteinase inhibitor	3	**2**/Chr 1, **1**/Chr 3
triosephosphate isomerase	3	**2**/Chr 1, **1**/Chr 9*

[a] Data were obtained using http://www.tigr.org/tigr-scripts/osa1web/gbrowse/rice/. Chr, chromosome. [b] The gene is chloroplastic.

vulnerability of this *lpa* mutant to certain pathogens or insect pests.

Bioinformatic Verification of Allergenic Proteins. To verify the presence of potential allergens, the Web server allergen predictor Evaller (http://bioinformatics.bmc.uu.se/evaller/) was used (*48*). This database predicts whether a protein sequence is likely to be allergenic or not on the basis of the presence of known allergenic peptides within its sequence. In the present study strong positive results were obtained for globulin, serpin, TPI, and glyoxalase putative dnaK-type molecular chaperone (BiP), endosperm lumenal binding protein, and enolase. The α-mylase inhibitor, on the other hand, gave a negative result, suggesting that this particular protein may not be allergenic.

Bioinformatic Verification of Detected Hypothetical Proteins. In the present study it was possible to identify the majority of differentially expressed proteins present in both XS-110 and *Os-lpa*-XS110-1. However, TurboSEQUEST identified three hypothetical proteins having functions that have not previously

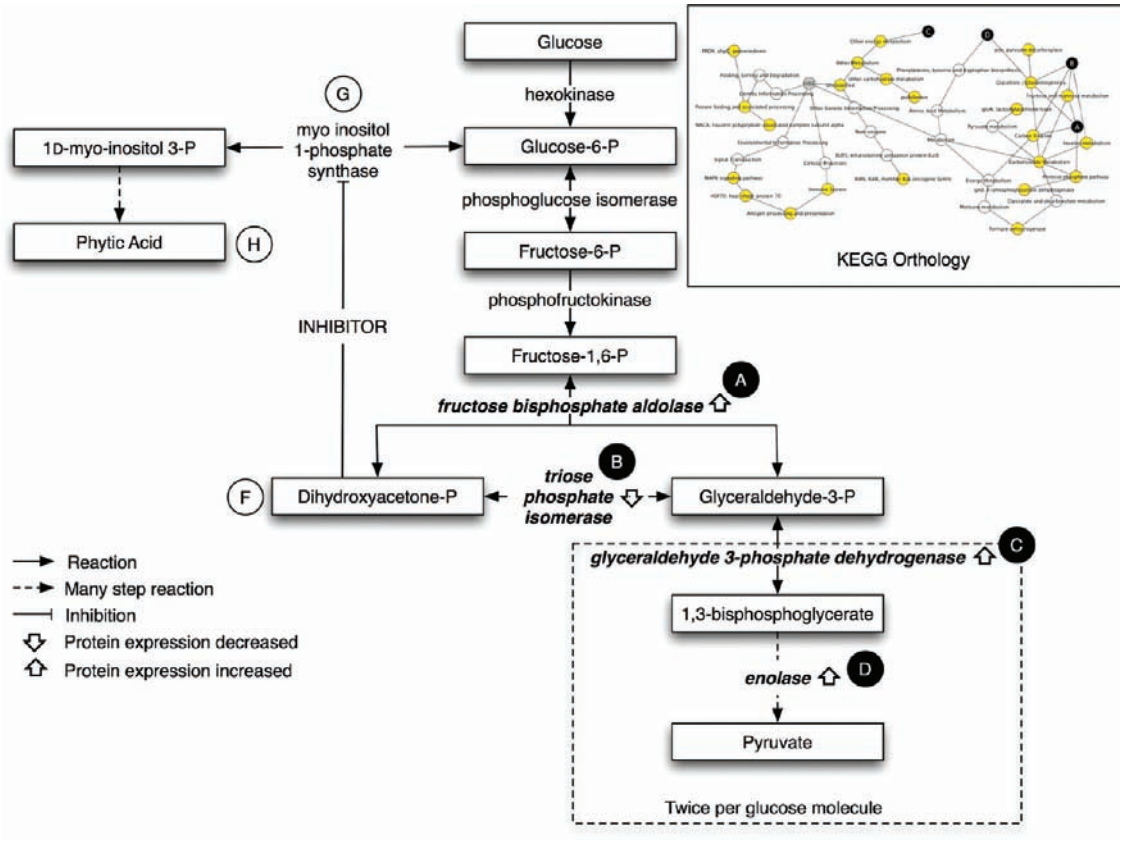

Figure 4. Possible metabolic regulation of phytic acid and KEGG orthology. The figure shows the glycolysis pathway from glucose through pyruvate. One critical step of the pathway is the cleavage of the six-carbon fructose-1,6-bisphosphate (fructose-1,6-P) by fructose bisphosphate aldolase (A). This results in the production of two three-carbon molecules, glyceraldehyde-3-phosphate (glyceraldehyde-3-P) and dihydroxyacetone phosphate (dihydroxyacetone-P; shown as F). Glyceraldehyde-3-phosphate is converted by glyceraldehyde 3-phosphate dehydrogenase (C) to 1,3-bisphosphoglycerate and then through a series of reactions, via enolase (D), to pyruvate. Dihydroxyacetone phosphate (F) is converted by triose-phosphate isomerase (B) to glyceraldehyde-3-phosphate and then to pyruvate as described above. Therefore, the reactions in the dotted box occur twice for each glucose molecule, yielding two pyruvates. Our findings have shown that the expressions of fructose bisphosphate aldolase (A), glyceraldehyde 3-phosphate dehydrogenase (C), and enolase (D) are all elevated and that the expression of triosephosphate isomerase (B) is decreased. This decrease in expression of triosephosphate isomerase (B) effectively means that the conversion of dihydroxyacetone phosphate (F) to glyceraldehyde-3-phosphate will be reduced; this will result in less flux through the pathway and a decrease in pyruvate production. The elevated levels of fructose bisphosphate aldolase (A), glyceraldehyde 3-phosphate dehydrogenase (C), and enolase (D) could be as a result of the cells trying to increase the level of pyruvate production. A failure of the conversion of dihydroxyacetone phosphate (F) to glyceraldehyde-3-phosphate would cause an intracellular increase in levels of dihydroxyacetone phosphate (F), which is a known inhibitor (*72*) of *myo*-inositol 1-phosphate synthase (G). This enzyme converts glucose 6-phosphate (glucose-6-P) to 1D-*myo*-inositol 3-phosphate (1D-myo-inositol 3-P), which is the start of the pathway to produce phytic acid (H). The box on the right presents part of the KEGG orthology networking raw data that resulted in understanding the relatedness of proteins that are involved in possible phytic acid biosynthesis. Proteins identified in this study as being involved in possible phytic acid biosynthesis are indicated by black circles and white letters.

Table 3. Potential Allergenic Proteins That Are Differentially Expressed in *Os-lpa*-XS110-1 and XS110-1[a]

potential allergen	biological activity	expression in *Os-lpa*-XS110-1
glyoxalase I (lactoylglutathione lyase) (*41*)	detoxification of methylglyoxal that is formed endogenously as a byproduct of the triosephosphate isomerase reaction in glycolysis (*68*)	↑
serine protease inhibitor (*35*)	plant defense against insect pests (*67*)	↑
globulin proteins (*32*, *33*)	storage proteins (*69*)	↓
bifunctional α-amylase/subtilisin inhibitors (*47*)	weak inhibitory activity toward some microbial α-amylases but not toward insects (*65*, *71*)	↓
triosephosphate isomerase; TPI (*66*)	sugar metabolism (*70*)	↓

[a] Up-regulated proteins in *lpa* line are indicated by "↑" and down-regulated proteins by "↓".

been annotated in the genome database. To date, the functions of these three proteins (Q94HJ5_ORYSA; Q6L578_ORYSA; Q7XP20) remain unknown. However, Q94HJ5_ORYSA has been annotated with the function "putative 3-β-hydroxysteroid dehydrogenase/isomerase protein", which is involved in metabolism of esterogen/androgen and C21-steroid hormone metabolism.

A BLAST search suggests that Q6L578_ORYSA has an N-terminal similarity to a DNA-binding protein. Furthermore, a reverse pattern-hit initiated (PHI) BLAST-based BLOCKS search (*49*) (http://blocks.fhcrc.org/) indicates that this region bears some similarity to a protein involved in meiotic chromosome segregation, w*apl* (*50*). BLAST searches show that Q7XP20 has a number of low-scoring hits to transcriptional regulator LacI family proteins. This family of proteins contains a helix−turn−helix (HTH) DNA binding domain and is present in many bacteria, controlling the expression of the Lac operon, although Q7XP20 does not align with these proteins in this domain. Furthermore, there are also low-scoring BLAST hits to histidine kinases. Thus, the identification of this particular protein has yet to be confirmed.

Role of Identified Proteins in Metabolism. Although the biosynthesis of phytic acid is not fully understood, it is well documented that it is made through phosphorylation of its backbone, *myo*-inositol (*51*−*53*). There is also a clear relationship between patterns of *myo*-inositol 1-phosphate synthase expression and the accumulation of phytic acid in rice (*54*). Reduced accumulation of phytate in *lpa* mutants seeds could be due to disturbance in conversion of *myo*-inositol and inorganic phosphorus (P_i) to phytic acid or alteration of regulatory intermediates (*20*). Liu et al. (*15*) mapped the *lpa* mutation in *Os-lpa*-XS110-1 to a region on the chromosome where a homologue of the *lpa3* maize *myo*-inositol kinase gene is located and suggested that *Os-lpa*-XS110-1 is an *lpa3* mutant similar to the *lpa3* mutants in maize that were previously identified by Shi et al. (*52*). Kim et al. (*55*) has suggested that a mutation of the rice *myo*-inositol kinase gene could lead to an *lpa* phenotype as in the maize *lpa3* mutation. Chemical profiling analyses showed that *Os-lpa-XS110-1* and the *lpa* line studied by Kim et al. (*55*) have very similar chemical alterations to *lpa3* mutants of maize, such as increased levels of *myo*-inositol and raffinose (*55*−*57*). Thus, it is highly likely that *Os-lpa*-XS110-1 is derived from mutation of the *myo*-inositol kinase gene.

In maize, the *lpa1* gene has been mapped to chromosome 1 by Raboy et al. (*12*), whereas in rice the *lpa1* locus is mapped to chromosome 2 (*14*). In *lpa1* and *lpa2*, alteration in *myo*-inositol phosphate synthase and inositol phosphate kinase functions, respectively, is suggested. In general, *lpa1* mutants have decreased levels of phytic acid-phosphorus (PA-P), with reductions ranging from 50 to 95% compared to levels present in the nonmutant seed, but high levels of P_i (*58*). Similarly, *lpa2* mutants are characterized by reduced levels of PA-P (up to 75%), but with high inositol phosphate and P_i contents in the seeds (*58*), whereas *lpa3* mutants are characterized by low levels of *myo*-inositol phosphate intermediates (*15*). Although *Os-lpa*-XS110-1 is very likely to be of the maize *lpa3* type, description of these mutations in maize may not necessarily be applicable to other crops (*15*). Hazebroek et al. (*57*) recently observed that *lpa3* maize kernels contained elevated levels of *myo*-inositol and raffinose (1.4 and 1.1 times higher, respectively, than the wild type), with even higher levels of ribose (*lpa3*:wild type was as high as 4.6) and galactose; interestingly, *Os-lpa*-XS110-1 rice seeds also contained significantly increased levels of galactose compared to the nonmutant (*21*). In general, accumulation of raffinosaccharides, as well as galactinol, in seeds is associated with the levels of *myo*-inositol, as has been observed in *lpa* barley mutants by Karner et al. (*59*). This same association between *myo*-inositol and raffinosaccharide levels in the seeds of *lpa* soybean mutants also occurs (*60*). However, Bowen et al. (*20*) did not find any significant changes in expression of genes in *lpa* barley that were directly involved in phytic acid biosynthesis; in their study the majority of identified genes in the mutant showed deceased expression levels, for example, genes involved in cell wall metabolism, cytokinin and ethylene signaling, and sugar transport. These inconsistent results between studies at the transcriptional and metabolome level may reflect the relatively low sensitivity of the selected method, especially when gene products are expressed at low levels. Interestingly, however, Bowen and colleagues observed a consistent decrease in transcript levels of a bifunctional α-amylase/subtilisin inhibitor over two consecutive years (*20*), which is consistent with the lower observed accumulation of this protein in *Os-lpa*-XS110 seeds compared to XS-110.

Recent microarray-based transcriptome studies suggest that mutagenesis of rice causes more changes in gene expression than transgene insertion, although both are likely to result in alteration of expression levels of nontargeted proteins (*61*).

In the present study no differentially expressed enzymes directly involved in phytic acid biosynthesis were detected. This finding was not unexpected because such enzymes are usually expressed during grain filling, rather than in the mature seeds. For example, the *myo*-inositol 1-phosphate synthase gene (MIPS1), which catalyzes the production of 1D-*myo*-inositol 3-phosphate from glucose 6-phosphate, is expressed at its highest level in rice grains 1 week after anthesis (*54*). Nevertheless, two differentially expressed enzymes with potential involvement in *myo*-inositol metabolism were identified. In the case of TPI, two spots, designated WT-350 and WT-359, were shown to be accumulated at levels 5- and 3-fold less, respectively, in *Os-lpa*-XS110-1 compared to XS-110 (**Table 1**), whereas FBA, represented by three spots, *lpa*-244, *lpa*-258, and *lpa*-937, was higher in *Os-lpa*-XS110-1 than in XS-110 (by 11.6-, 8.6-, and 3.2-fold, respectively; **Table 1**). These two enzymes are involved in many metabolic pathways including inositol metabolism, fructose−mannose metabolism, glycolysis, carbon fixation, and the pentose−phosphate pathway. TPI specifically catalyzes the interconversion of glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), whereas FBA produces GAP and

DHAP from fructose 1,6-bisphosphate (F1,6P). The TPI reaction occurs at the important metabolic branch points between gluconeogenesis, glycolysis, the pentose pathway, the methylglyoxal pathway, and glycerol production/utilization. Thus, decreases in TPI levels could have a considerable consequence for energy homeostasis in the cell as the production of pyruvate from the conversion of GAP would be reduced as a consequence of the conversion of DHAP to GAP being altered. This potential shortfall in energy within the cells may be the reason for the lower germination rates, and subsequent slower growth rates, for *Os-lpa*-XS110-1 compared to the XS-110, as previously reported (*15*). Interestingly, DHAP and GAP are inhibitors of *myo*-inositol-3 phosphate synthase (**Figure 4**), which catalyzes the conversion of glucose 6-phosphate (G6P) to *myo*-inositol 3-phosphate (MIP), the rate-limiting step in inositol synthesis. Therefore, the decreased levels of TPI, coupled with increased levels of FBA, as seen in *Os-lpa*-XS110-1, will result in inositol being limited, which in turn may explain the decrease in the levels of phytic acid in this particular line (**Figure 4**). The proposed mechanism would be that elevated levels of FBA cause an increase in the levels of GAP and DHAP. GAP is utilized in glcolysis, etc., whereas DHAP accumulates due to decreased levels of TPI, which would convert it to GAP. The DHAP then inhibits MIP, thus decreasing inositol synthesis.

The differential expression of proteins observed in this study between the *lpa* line and its parent could be due to a number of factors. Some differences may be a consequence of the phenotypic appearance of nontargeted random mutations. Because *Os-lpa*-XS110-1 was developed through γ irradiation, it is expected that mutations other than the *lpa* gene also occurred to the genome. Till et al. (*62*) reported that methane ethyl methanesulfonate mutagenesis in rice can generate a mutation density of about one DNA lesion per 300 kb. Some of these DNA lesions can translate into mutations at the protein level and can be detected with proteomic techniques such as those used in the present study. Other observed differences could represent the effect of *lpa* mutation on the expression of other related genes, such as TPI and FBA, that were discussed above. Yet a further source of difference in protein accumulation in the seeds could be a consequence of genetic heterozygosity in the parent line when it was mutagenized, which could lead to differences between the parental line and its progenies, independent of mutagenesis. The existence of such a genetic residue in XS-110 has already been demonstrated using microsatellite analysis (*63*); thus, its contribution to these observed differences in the proteome between the two lines cannot be ruled out. A recent study by Ahn et al. (*64*) demonstrated that the location where a plant is grown may have a greater effect on accumulation and storage of total P and phytate P than the variety of rice itself. It is therefore possible that some of the changes observed in the present study may not have been detected if the plants had been grown at different geographic/climatic locations or in a different season.

Results from the present study demonstrate that SELDI-TOF profiling is an efficient way of searching for potential biomarkers to differentiate between different crop lines. Furthermore, this technology provides a rapid and sensitive screening method, particularly for low molecular weight proteins that may escape detection by other separation techniques such as 2-DE. 2-DE, on the other hand, is a robust method for separating a broader dynamic range of proteins for subsequent identification by mass spectrometry. Using these two complementary approaches we could readily differentiate between the novel *Os-lpa*-XS110-1 and XS-110, in addition to identifying the differentially expressed proteins, including potential allergens. However, the occurrence of potential allergens in both *Os-lpa*-XS110-1 and XS-110 does not necessarily imply that one line is any more allergenic than the other.

Detailed analysis of the genome, proteome, and metabolome is required to better understand the effect of changes to the phenotype and genotype and of any potential benefits/risks to consumers. Because the major objective of the present study was to investigate the robustness of proteome profiling and bioinformnatics as tools for safety assessment of novel foods, rather than in testing the safety of low phytic acid rice per se, experimental plants were grown at only a single location. However, as part of a comprehensive assessment of the safety of *lpa* crops, further studies would be required whereby the novel lines were grown in different geographic and climatic regions and over different seasons and years to determine possible environmental effects on gene expression.

NOTE ADDED AFTER ASAP PUBLICATION

Changes to the reference citations and Tables 1 and 3 have been made after the ASAP publication of May 4, 2010. These corrections are reflected in the ASAP publication of May 7, 2010.

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