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Impact of Cross-Breeding on the Contents of Inositol Phosphate Isomers and the Metabolite Profiles of *Low Phytic Acid* Soybean (*Glycine max* (L.) Merr.) Mutants

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Dedicated to my beloved father Hubert Anton Goßner

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ABBREVIATIONS

2-PGK	2-Phosphoglycerate kinase
ABC	ATP-binding cassette
AES	Atomic emission spectroscopy
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
ATP	Adenosine triphosphate
CADMA	Competitive amplification of differentially melting amplicons analysis
CE	Capillary electrophoresis
CI	Chemical ionization
CITP	Capillary isotachophoresis
ClC-3	Chloride channel 3
CZE	Capillary zone electrophoresis
Da	Dalton
EI	Electron ionization
ESI	Electrospray ionization
FAME	Fatty acid methyl ester
FFA	Free fatty acids
FID	Flame ionization detection
FT-ICR	Fourier transform ion cyclotron resonance
GABA	*γ*-Aminobutyric acid
GC	Gas chromatography
glog	Generalized logarithm
GM	Genetically modified
HCA	Hierarchical cluster analysis
HILIC	Hydrophilic interaction liquid chromatography
HM	Homozygous *lpa* mutant
HPIC	High pressure ion chromatography
HPTLC	High-performance thin-layer chromatography
HRM	High-resolution melting
HSD	Honestly significant difference
HWT	Homozygous wild-type

ICP	Inductivity coupled plasma
IMP	Inositol monophosphate phosphatase
Ins PolyP K/P	Inositol polyphosphate kinase/ phosphatase
$InsP_2$−$InsP_5$	Lower inositol phosphates
$InsP_6$	Phytic acid
IPK1	Inositol 1,3,4,5,6-penta*kis*phosphate 2-kinase
ITPK	Inositol 1,3,4-triphosphate 5-/6-kinase
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
lpa	Low phytic acid
LSD	Fisher's least significant difference
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MIK	*Myo*-inostitol kinase
MIPS1	D-*myo*-inositol 3-phosphate synthase gene 1
MRP	Multi-drug resistance-associated protein
MS	Mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide
NK	Neutral killer
NMR	Nuclear magnetic resonance spectroscopy
NP	Normal-phase
OPLS-DA	Orthogonal partial least squares-discriminant analysis
OSC	Orthogonal signal correction
PC	Principal component
PCA	Principal component analysis
PI3K/Akt	Phosphoinositide 3-kinase/serine/threonine protein kinase B
PLC	Phospholipase C
PLS-DA	Partial least square discriminant analysis
PP-$InsP_s$	Inositol pyrophosphates
PtdI4K	Phosphatidyl inositol 4-kinase
PtdIns	Phosphatidylinositol
PtdIP5K	Phosphatidyl inositol 4-phosphate 5-kinase
PTFE	Polytetrafluoroethylene

Q	Quadrupole
QTL	Quantitative trait loci
RF	Radio frequency
RFO	Raffinose family oligosaccharide
ROS	Reactive oxygen species
RP	Reversed-phase
RRT	Relative retention time
RSD	Relative standard deviation
SD	Standard deviation
SIMCA	Soft independent modeling of class analogy
SOM	Self-organizing map
TOF	Time of flight
TWSS	Total water-soluble sugar
UV	Ultraviolet
VIP	Variable importance in projection
WSP	Water-soluble protein
WT	Wild-type

SUMMARY

Most of the inorganic phosphorus in mature seeds is stored in the form of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, $InsP_6$) which is a strong chelator of cationic metal micronutrients and therefore considered as an antinutrient in food and feed. In addition, undigested phytate in animal waste contributes to phosphorus pollution and eutrophication of waterways. One approach to overcome these nutritional and environmental problems associated with $InsP_6$ is the generation of *low phytic acid* (*lpa*) crops. Such *lpa* mutants often show inferior agronomic performance, and cross-breeding with commercial cultivars is being applied to improve not only their nutritional but also their agronomic characteristics. However, the consequences of such crossing steps on the contents of $InsP_6$, lower inositol phosphate isomers ($InsP_2$-$InsP_5$) and the metabolite profiles of the resulting homozygous *lpa* soybean progenies were unknown.

Therefore, the previously developed *MIPS1 lpa* soybean mutants *Gm-lpa*-TW-1 and *Gm-lpa*-TW-1-M as well as the *IPK1 lpa* soybean mutant *Gm-lpa*-ZC-2 were crossed with three commercial wild-type (WT) cultivars or among themselves, and the $InsP_6$ and $InsP_2$-$InsP_5$ contents in the resulting homozygous *lpa* mutant and homozygous WT progenies of various generations and from different growing seasons were determined via high pressure ion chromatography (HPIC). For the *MIPS1* mutant, the *lpa* trait was not changed by cross-breeding with a WT cultivar, and *lpa* progenies showed $InsP_6$ reductions of about 44% compared to WT progenies. In contrast, *IPK1* progenies exhibited distinct accumulations of specific $InsP_3$-$InsP_5$ isomers (up to 12.4 mg/g) in comparison to the progenitor *lpa* mutant (4.7 mg/g). The magnitude of $InsP_6$ reduction in *IPK1* mutant progenies varied between 43% and 71% and was dependent on the WT crossing parent. Double *lpa* mutants simultaneously carrying the *IPK1* and *MIPS1* mutation target exhibited the highest $InsP_6$ reductions up to 87%, with only moderate accumulations of $InsP_3$-$InsP_5$ (2.5 mg/g). Overall, the homozygous *lpa* mutant soybean progenies always displayed significantly lower contents of $InsP_6$ than the original WTs subjected to the mutations, independent from cross-breeding steps, crossing parents or environmental influence. This makes cross- and selection-breeding a useful tool to generate new *lpa* soybean cultivars with the intended *lpa* trait.

In addition, the *lpa* soybean mutant *Gm-lpa*-TW-1-M, resulting from a 2 bp deletion in the third exon of the *myo*-inositol 3-phosphate synthase (*MIPS1*) gene, was used as

an example to investigate the stability of the mutation-induced metabolic changes in homozygous *lpa* mutant and homozygous WT progenies resulting from the cross-breeding with a commercial WT cultivar. The application of a non-targeted GC-based metabolite profiling approach allowed the analysis of a broad range of lipophilic and polar low molecular weight soybean constituents including fatty acid methyl esters, free fatty acids, fatty alcohols, tocopherols, phytosterols, sugars, sugar alcohols, acids, amino acids, amines, and organic as well as inorganic acids. Multivariate und univariate statistical approaches revealed that the *MIPS1* mutation led not only to the intended effect of $InsP_6$ reduction in homozygous *lpa* mutant progenies but also to pronounced metabolic changes of other nutritionally important constituents. In comparison to the respective WT progenies, *lpa* mutant progenies exhibited reduced contents of raffinose oligosaccharides and galactosyl cyclitols as well as increased concentrations of sucrose and various free amino acids. This *MIPS1*-induced metabolite signature was consistently expressed over generations and different growing seasons despite the cross-breeding step. This indicated that not only the primary *MIPS1* soybean mutants but also their homozygous *lpa* mutant progenies might be valuable genetic resources for the commercial breeding of *lpa* soybean seeds which combine the advantages of increased mineral bioavailability, reduced phytate-related environmental problems as well as enhanced metabolizable energy and carbohydrate digestibility.

The elaborated data demonstrated that cross-breeding of *lpa* soybean mutants with commercial cultivars may be a valuable tool to modulate the contents of both phytic acid and lower inositol phosphates in homozygous *lpa* mutant progenies and thus to further improve their nutritional quality. The generation of *lpa* double mutants offers the potential to drastically reduce the $InsP_6$ content in soybean seeds. Cross-breeding was shown to neither compromise the intended effect of $InsP_6$ reduction nor the *MIPS1* mutation-specific metabolite signature of homozygous *lpa* mutant soybean progenies. Therefore, cross-breeding was shown to be a useful strategy to generate *lpa* soybean seeds stably exhibiting improved nutritional traits.

ZUSAMMENFASSUNG

In reifen Samen wird der Großteil des anorganischen Phosphors in Form von Phytinsäure (*myo*-Inositol-1,2,3,4,5,6-hexa*kis*phosphat, $InsP_6$) gespeichert, die ein starker Chelatbildner von kationischen Metallmikronährstoffen ist und daher als antinutritive Substanz in Lebens- und Futtermitteln gilt. Darüber hinaus trägt unverdautes Phytat in Gülle zur Phosphorverschmutzung und zur Eutrophierung von Gewässern bei. Ein Ansatz zur Lösung dieser mit $InsP_6$ assoziierten Ernährungs- und Umweltprobleme ist die Erzeugung von Kulturpflanzen mit erniedrigten Phytinsäuregehalten (*low phytic acid, lpa*). Solche *lpa* Mutanten weisen jedoch häufig schlechtere agronomische Leistungen auf und werden daher mit kommerziellen Sorten gekreuzt, um sowohl ihre ernährungsphysiologischen als auch ihre agronomischen Eigenschaften zu verbessern. Die Auswirkungen solcher Kreuzungsschritte auf die Gehalte an $InsP_6$, niederphosphorylierte Inositolphosphat-Isomere ($InsP_2$-$InsP_5$) und die Metabolitenprofile der resultierenden homozygoten Nachkommen wurden bisher allerdings nicht untersucht.

Daher wurden die zuvor entwickelten *MIPS1 lpa* Sojamutanten *Gm-lpa*-TW-1 und *Gm-lpa*-TW-1-M sowie die *IPK1 lpa* Sojamutante *Gm-lpa*-ZC-2 mit drei kommerziellen Wildtyp (WT) Sorten oder untereinander gekreuzt und die $InsP_6$ sowie die $InsP_2$-$InsP_5$ Gehalte in den resultierenden homozygoten *lpa* und homozygoten WT Nachkommen verschiedener Generationen und Anbauzeiten mittels Hochdruck-Ionenchromatographie (HPIC) untersucht. Das *lpa* Merkmal der *MIPS1* Mutante wurde durch das Einkreuzen einer WT Sorte nicht verändert; *lpa* Nachkommen wiesen $InsP_6$ Abnahmen von etwa 44% im Vergleich zu WT Nachkommen auf. Im Gegensatz dazu zeigten *IPK1* Nachkommen deutliche Anreicherungen spezifischer $InsP_3$-$InsP_5$ Isomere (bis zu 12,4 mg/g) im Vergleich zur ursprünglichen *lpa* Mutante (4,7 mg/g). Das Ausmaß der $InsP_6$ Reduktion in *IPK1* Nachkommen schwankte zwischen 43% und 71% und war abhängig vom WT Kreuzungspartner. *Lpa* Doppelmutanten, die gleichzeitig die *IPK1* und *MIPS1* Mutationen trugen, zeigten mit bis zu 87% die höchsten $InsP_6$ Abnahmen, begleitet von nur mäßigen Anreicherungen an $InsP_3$-$InsP_5$ (2,5 mg/g). Insgesamt wiesen die homozygoten *lpa* Nachkommen unabhängig von den Kreuzungen, den Kreuzungspartnern oder Umwelteinflüssen einen deutlich niedrigeren $InsP_6$ Gehalt auf als die Wildtypen, die ursprünglich den Mutationen unterzogen worden waren. Dies

verdeutlichte, dass Kreuzungs- und Selektionszüchtung eine nützliche Strategie bei der Erzeugung von neuartigen *lpa* Sojasorten darstellt.

Darüber hinaus wurde die *lpa* Sojamutante *Gm-lpa*-TW-1-M, die auf eine 2-bp-Deletion im dritten Exon des *myo*-Inositol 3-Phosphat Synthase Gens (*MIPS1*) zurückzuführen ist, als Beispiel herangezogen, um nach dem Einkreuzen einer WT Sorte die Stabilität der mutationsbedingten metabolischen Veränderungen in homozygoten *lpa* und homozygoten WT Nachkommen zu untersuchen. Die Anwendung eines nicht zielgerichteten GC-basierten Metabolite Profiling Ansatzes ermöglichte die Analyse eines breiten Spektrums an niedermolekularen lipophilen und polaren Verbindungen, darunter Fettsäuremethylester, freie Fettsäuren, Fettalkohole, Tocopherole, Phytosterole, Zucker, Zuckeralkohole, Säuren, Aminosäuren, Amine und organische sowie anorganische Säuren. Multivariate und univariate statistische Auswertungen ergaben, dass die *MIPS1* Mutation nicht nur zur beabsichtigten $InsP_6$ Reduktion in den homozygoten *lpa* Nachkommen führte, sondern darüber hinaus zu ausgeprägten metabolischen Veränderungen anderer ernährungsphysiologisch relevanter Komponenten. So zeigten *lpa* Nachkommen im Vergleich zu den WT Nachkommen reduzierte Gehalte an Raffinose-Oligosacchariden und Galactosylcyclitolen sowie erhöhte Konzentrationen an Saccharose und verschiedenen freien Aminosäuren. Diese *MIPS1*-spezifische Metaboliten-Signatur blieb trotz des Kreuzungsschrittes über Generationen und zu unterschiedlichen Anbauzeiten erhalten. Die Ergebnisse deuten darauf hin, dass nicht nur die primären *MIPS1* Sojamutanten, sondern auch ihre homozygoten *lpa* Nachkommen, wertvolle genetische Ressourcen für die kommerzielle Züchtung von *lpa* Sojabohnen darstellen. Sie verbinden die Vorteile einer erhöhten Mineralstoffbioverfügbarkeit mit reduzierten Phytat-bedingten Umweltproblemen sowie einem erhöhten metabolisierbaren Energieanteil und verbesserter Kohlenhydratverdaulichkeit.

Die erarbeiteten Daten zeigten, dass die Kreuzung von *lpa* Sojamutanten mit kommerziellen Sorten eine wertvolle Methode darstellen kann, um die Gehalte an Phytinsäure und niederphosphorylierten Inositolphosphaten in homozygoten *lpa* Nachkommen zu modulieren und dadurch ihre Nährstoffqualität weiter zu verbessern. Die Erzeugung von *lpa* Doppelmutanten bietet darüber hinaus die Möglichkeit, den Phytinsäuregehalt in Sojabohnen drastisch zu reduzieren. Es wurde gezeigt, dass Kreuzungen weder die beabsichtigte Wirkung der $InsP_6$ Reduktion noch die *MIPS1*-

spezifische Metaboliten-Signatur in homozygoten *lpa* Sojanachkommen beeinträchtigen. Daher stellt Kreuzungszüchtung eine nützliche Strategie dar, um *lpa* Sojamutanten mit stabilen und ernährungsphysiologisch wertvollen Eigenschaften zu erzeugen.

1 INTRODUCTION AND OBJECTIVES

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, $InsP_6$) represents the major storage form of inorganic phosphorus (P) in mature seeds such as grains, oilseeds, nuts and legumes (Raboy, 1997). Owing to its ability to form indigestible chelates with cationic metal micronutrients like zinc and iron, thereby limiting their bioavailability, phytic acid is considered as an antinutrient in food and feed (Kumar *et al.*, 2010). In addition, excreted phytate in the manure of monogastric animals is degraded by natural soil microorganisms and thus contributes to P pollution and eutrophication of waterways (Leytem and Maguire, 2007). Various efforts have been made to overcome these obstacles by generating *low phytic acid* (*lpa*) crops comprising soybean (Yuan *et al.*, 2007), common bean (Campion *et al.*, 2009), maize (Shi *et al.*, 2007), rice (Ali *et al.*, 2013), wheat (Guttieri *et al.*, 2004), and barley (Rasmussen and Hatzack, 1998) using *γ*-irradiation, chemically induced mutagenesis or genetic engineering.

Soybean (*Glycine max* (L.) Merr.), the most important cultivated legume worldwide (Stagnari *et al.*, 2017), has a multitude of uses in food, feed and industrial products (Liu, 1997a). Phytic acid contents up to 4.6% have been reported in soybeans (Kumar *et al.*, 2005). By generating *lpa* soybean mutants, phytic acid reductions of up to 80% compared to their respective wild-type (WT) cultivars were accomplished (Wilcox *et al.*, 2000). *Lpa* mutants often possess inferior agronomic performance (Raboy, 2007), and cross- and selection-breeding is being applied to improve both their agronomic and nutritional characteristics (Spear and Fehr, 2007; Zhao *et al.*, 2008). The effects of crossing an *lpa* soybean mutant with either a commercial wild-type cultivar or another *lpa* mutant carrying a different mutation target on the contents of phytic acid and lower inositol phosphate isomers in the resulting progenies have not been investigated so far. Therefore, the previously developed *MIPS1 lpa* soybean mutants *Gm-lpa*-TW-1 (TW-1-*lpa*) and *Gm-lpa*-TW-1-M (TW-1-M-*lpa*) as well as the *IPK1 lpa* mutant *Gm-lpa*-ZC-2 (ZC-*lpa*) were crossed with commercial wild-type cultivars, and the phytic acid and lower inositol phosphate contents in the resulting homozygous WT and homozygous *lpa* mutant progenies of various generations grown at different locations were determined via high-pressure ion chromatography (HPIC). The objectives of the first part of this study were: (i) to assess the impact of cross-breeding of the *MIPS1* and *IPK1* mutants with commercial cultivars on the intended effect of phytic acid reduction and the occurrence of lower inositol phosphate isomers in the resulting progenies, and (ii) to investigate the extent of phytic acid reduction and the

effects on the lower inositol phosphate isomers' contents in homozygous *lpa* double mutants simultaneously carrying the *IPK1* and *MIPS1* mutation target.

Previous investigations have demonstrated that induced mutations lead not only to the intended effect of phytic acid reduction in *lpa* mutants but can also result in changes of other nutritionally relevant metabolites (Frank *et al.*, 2007; Frank *et al.*, 2009; Hitz *et al.*, 2002). For instance, a GC-based metabolite profiling approach revealed consistent changes in the concentrations of *myo*-inositol, raffinose oligosaccharides (RFOs), galactosyl cyclitols and sucrose in the primary *MIPS1* soybean mutation TW-*lpa* compared to its wild-type cultivar Taiwan 75 (Frank *et al.*, 2009). The stability of such mutation-induced metabolic signatures of *lpa* mutants upon cross-breeding have so far only been investigated in rice (Zhou *et al.*, 2018). Therefore, in the second part of this work, the *MIPS1* mutant TW-1-M-*lpa* was used as an example to study the mutation-induced metabolic changes in progenies resulting from the crossing with a commercial cultivar by a GC-based metabolite profiling approach allowing the analysis of a broad array of low molecular weight constituents. The objectives of this part of the study were: (i) to investigate the impact of cross-breeding of the *MIPS1 lpa* mutant TW-1-M-*lpa* with a commercial cultivar on the mutation-induced metabolite signature of the resulting homozygous *lpa* mutant progenies and (ii) to evaluate the stability of the *lpa* trait in crossbred progenies depending on generations and growing seasons.

The present thesis is structured as follows:

- After these introductory remarks describing the objectives of the study (Chapter 1), the background and methodologies of the thesis are presented in Chapter 2.
- Materials and methods, results of the studies and their specific discussions in the light of existing literature are presented in the two original publications included in Chapter 3.
- An overall discussion section across the presented dissertation topics depicting the significance of the elaborated results in the light of existing literature knowledge is presented in Chapter 4.

2 BACKGROUND

2.1 Soybean (*Glycine max* (L.) Merr.)

Soybean is an annual plant which was domesticated in northeastern China during 1700-1100 B.C (Singh and Hymowitz, 1999). Taxonomically, it belongs to the legume family *Leguminosae* or *Fabaceae*, subfamily *Papilionoideae*, tribe *Phaseoleae* and the genus *Glycine*, which contains two subgenera, *Soja* (annuals) and *Glycine* (perennials). The subgenus *Glycine* consists of 22 recognized species, and the subgenus *Soja* contains the two subspecies *Glycine max* (L.) Merr. subsp. *max*, the cultivated soybean, and its progenitor *Glycine max* subsp. *soja* (Siebold & Zucc.) H. Ohashi, the wild soybean (Poehlman and Sleper, 1995; Pratap *et al.*, 2015).

2.1.1 Production, processing and utilization

Soybean is the dominant oil-seed contributing to about 55% of global oilseed production followed by rapeseed, cotton, and peanut with 15%, 10% and 9%, respectively (Pratap *et al.*, 2015). In 2017, the worldwide cultivated area of soybean reached 124 million ha with a global soybean production amounting to 353 million tons, of which 312 million tons were produced in the Americas and 11 million tons in Europe (FAO, 2019). The United States of America, Brazil and Argentina are the world's main soybean producers and contribute to more than 80% of the global soybean production (Table 1). The best growing conditions for soybean are temperate and subtropical climates with a temperature ranging from 25 to 32 °C and moderate moisture with 400-800 mm rainfall. The average production cycle of soybean from planting to harvesting is 90-110 days (Nwokolo, 1996). As a legume crop, soybean is able to fix atmospheric nitrogen by establishing a symbiotic relationship with *Rhizobium* species in root nodules (Zahran, 1999). Thereby, soybean can fix up to 450 kg N/ha, covering on average 50-60% of its nitrogen demand (Salvagiotti *et al.*, 2008) making this crop less dependent on synthetic nitrogen fertilization.

Due to its high content in nutrients, soybean has a multitude of uses in foods, feed, industrial products and consumables and it is a source of biofuel and industrial oils (Hartman *et al.*, 2011). In the USA, only about 5% of the produced soybean seeds are consumed by humans whereas the bigger part is crushed for the production of soybean oil and meal (Nwokolo, 1996).

Table 1. Production, area and yield of soybean in the main producing countries worldwide in 2017 (FAO, 2019).

	Production (million t)	Area harvested (million ha)	Yield (t/ha)	Worldwide production (%)
USA	119.5	36.2	3.30	33.9
Brazil	114.6	33.9	3.38	32.5
Argentina	55.0	17.3	3.17	15.6
China	13.2	7.3	1.79	3.7
India	11.0	10.6	1.04	3.1
Paraguay	10.5	3.4	3.10	3.0

Solvent extraction, e.g. with hexane, is used to obtain crude oil and defatted meal from soy flakes (Proctor, 1997). About 98% of the defatted soybean meal is employed as high protein source in animal feed, e.g. for poultry, pork and aquaculture species (Goldsmith, 2008; Hartman *et al.*, 2011). Commercial forms of soybean protein include defatted soy flours, flakes or grits, soy concentrates and soy isolates with protein contents of about 50%, 70% and 90%, respectively (Wolf, 1970).

To remove impurities from crude soy oil, refining processes including degumming, alkali refining, bleaching, and deodorization are used. To further modulate the physicochemical properties of the oil for the use in certain food processes, hydrogenation, interesterification, winterization and fractionation can be applied (Proctor, 1997). About 95% of the soy oil is used as edible oil, e.g. for the manufacturing of mayonnaise, salad dressings, margarine and shortening. A small share is used in industries related to the production of pharmaceuticals, plastics, papers, inks, paints, polishers, pesticides, and cosmetics (Hartman *et al.*, 2011; Pratap *et al.*, 2015). Only in the USA, relevant quantities of soybean oil are used at a commercial scale to produce biodiesel by transesterification (Pratap *et al.*, 2015). Furthermore, soybean oil is the major source for lecithin, phosphatides obtained as by-product in the soybean oil degumming process. Lecithin is added to foods, feedstuff, cosmetics and pharmaceuticals as emulsifier, wetting and dispersing or softening agent and as antioxidant (Erickson, 1995).

Traditional soyfoods produced of whole soybean for human consumption can be classified into non-fermented and fermented products. Non-fermented soyfoods include tofu, soy sprouts, soymilk, yuba (soymilk film), okara (soy pulp), vegetable

soybeans, soynuts and toasted soy flour. Fermented soyfoods comprise soy sauce, miso (fermented soy paste), natto (*Bacillus natto* fermented whole soybeans), tempeh (*Rhizopus* mold fermented, cake-like soybeans), soy yoghurt, sufu (fermented tofu), and douchi (fermented and salted black soybeans) (Liu, 2008).

2.1.2 Seed composition

Soybean seeds stand out from other legume seeds due to their unique chemical composition with high protein contents of around 40% and oil contents of about 20% (Liu, 1997a). The mean composition of mature and raw soybean seeds is summarized in Table 2.

More than 70% of the global humans' protein consumption originates from plants, whereof about 50% comes from soybean (Bao *et al.*, 1993). The two major soybean proteins in seed storage globulins are glycinin and *β*-conglycinin, accounting for about 40% and 30% of the total seed protein, respectively (Krishnan and Nelson, 2011). The soy protein contains all essential amino acids (Table 2B); however, the concentrations of the sulfur-containing amino acids (cysteine and methionine) are rather low. On the other hand, soy protein contains remarkable contents of lysine, an essential amino acid which is limiting in most cereal proteins. Therefore, soybean might be a good protein source to improve the total protein status of populations depending on cereal grains as stable foods because soybean and cereals are complementary for lysine and methionine (Nwokolo, 1996).

The most abundant fatty acid esterified in soybean triacylglycerols is linoleic acid (C18:2; 54%), followed by oleic acid (C18:1; 23%), palmitic acid (C16:0; 11%), linolenic acid (C18:3; 7%), stearic acid (C18:0; 4%) and minor amounts of palmitoleic (C16:1; < 1%) and myristic acid (C14:0; < 1%) (Table 2C). On the one hand, the high contents of the essential unsaturated fatty acids C18:2 and C18:3 add to soybean's high nutritional value but, on the other hand, make it susceptible to oxidation and off-flavor development. The most abundant phytosterols found in soybean seeds are campesterol, stigmasterol, and *β*-sitosterol (Medic *et al.*, 2014). Phospholipids are the most important polar lipids in crude soybean oil with contents of 2-3.5% (Nwokolo, 1996). They mainly consists of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol and phosphatidic acid (Liu, 1997a).

Table 2. Composition of mature and raw soybean seeds. Contents of A, proximates; B, amino acids; C, lipids; D, minerals and E, vitamins (USDA National Nutrient Database for Standard Reference, 2019).

(A) Proximates	amount in 100 g
Water [g]	8.5
Energy [kcal]	446
Protein [g]	36.5
Lipids [g]	19.9
Carbohydrates [g]	30.2
Dietary fiber [g]	9.3
Sugar [g]	7.3
Ash [g]	4.9

(B) Amino acids	amount in 100 g
Tryptophan* [g]	0.59
Threonine* [g]	1.77
Isoleucine* [g]	1.97
Leucine* [g]	3.31
Lysine* [g]	2.71
Methionine* [g]	0.55
Cysteine [g]	0.66
Phenylalanine* [g]	2.12
Tyrosine [g]	1.54
Valine* [g]	2.03
Arginine [g]	3.15
Histidine [g]	1.10
Alanine [g]	1.92
Aspartic acid [g]	5.11
Glutamic acid [g]	7.87
Glycine [g]	1.88
Proline [g]	2.38
Serine [g]	2.36

* essential amino acids

(C) Lipids	amount in 100 g
Fatty acids	
Saturated [g]	2.89
C14:0 [g]	0.06
C16:0 [g]	2.12
C18:0 [g]	0.71
Monounsaturated [g]	4.40
C16:1 [g]	0.06
C18:1 [g]	4.34
Polyunsaturated [g]	11.26
C18:2** [g]	9.93
C18:3** [g]	1.33
Phytosterols [mg]	161

**essential fatty acids

(D) Minerals	amount in 100 g
Calcium [mg]	277
Iron [mg]	15.7
Magnesium [mg]	280
Phosphorus [mg]	704
Potassium [mg]	1797
Sodium [mg]	2.0
Zinc [mg]	4.9
Copper [mg]	1.7
Manganese [mg]	2.5
Selenium [µg]	17.8

(E) Vitamins	amount in 100 g
Vitamin C [mg]	6.0
Thiamin [µg]	870
Riboflavin [µg]	870
Niacin [mg]	1.62
Pantothenic acid [µg]	790
Vitamin B_6 [µg]	380
Folate [µg]	375
Vitamin A [IU]	22
Vitamin E [µg]	850
Vitamin K [µg]	47

The third abundant component in soybean seeds are carbohydrates with contents up to 35% dry matter (Medic *et al.*, 2014). They consist of about 50% non-structural and structural carbohydrates, respectively. Structural carbohydrates or cell wall polysaccharides include cellulose, hemicellulose and pectins, mannans, galactans, and xyloglucans (Karr-Lilienthal *et al.*, 2005). Non-structural carbohydrates comprise sucrose (1.1-7.4% dry weight), stachyose (1.2-6.9%), and raffinose (0.1-1.4%), starch (0.2-1.0%) or fructose (0.03-2.5%), glucose (0.03-2.4%), and maltose (0.3-0.5%) (Medic *et al.*, 2014).

Besides proteins, lipids and carbohydrates, soybean also contains various minor compounds such as minerals, vitamins and phenols. Like most other legumes, soybean is rich in potassium but low in sodium (Table 2D). In addition, soybean is a valuable source of thiamine, riboflavin and niacin (Table 2E) besides phosphorus, although a significant amount of phosphorus is stored in the form of phytic acid which thereby has only limited bioavailability (Nwokolo, 1996).

In addition, soy protein-containing foods are a good source of isoflavones, a group of phytoestrogens with the aglycones genistein, daizein and glycitein as major representatives (Liu, 1997a). In soybean, contents up to about 420 mg isoflavones/100 g have been described (Wang and Murphy, 1994). Isoflavones have been reported to provide antioxidant and antifungal activity, to lower blood cholesterol, inhibit bone resorption and act as anticarcinogens and thereby potentially lowering the risk for diseases such as cancer, osteoporosis, or heart and kidney disease (Liu, 1997a; Messina, 1997).

Despite its high nutritional value, soybean also contains natural antinutrients, among them are heat-labile compounds, such as protease inhibitors, lectins, goitrogens and phytate, as well as heat-stable compounds, such as flatulence–producing oligosaccharides and saponins (Liener, 1981).

2.1.3 Breeding

The major soybean breeding objectives are improvement of seed yield, maturity for area of production, resistance to shattering and lodging, tolerance to environmental stresses, disease resistance, insect resistance, and seed quality parameters such as protein or oil content (Poehlman and Sleper, 1995). Both the wild and the cultivated soybeans are palaeopolyploid with 2n = 40 chromosomes; in total, the soybean genome consists of 1.1 giga base pairs (Pathan and Sleper, 2008).

About 170,000 *Glycine max* accessions are maintained in 70 countries, with China and the United Stated possessing the largest collections with nearly 26,000 and 19,000 *Glycine max* accessions, respectively (Pathan and Sleper, 2008). Up to now, 173 soybean mutant varieties have been developed by mutation breeding and have been officially or commercially released (FAO/IAEA, 2019).

Breeding methods of crop plants comprise recurrent selection, hybridization, mutation breeding, the use of molecular markers, and biotechnological approaches (Liu, 1997b). Conventional breeding involves the phenotypic selection of superior individuals from segregating populations and crossing among the selected genotypes (Liu, 1997b). However, these processes are time-consuming; 8-10 years are necessary until a variety can be released (Pathan and Sleper, 2008). Conventional breeding has had a remarkable impact on the yield increase of soybean since 1930 (Pathan and Sleper, 2008). Soybean is usually a self-pollinated crop with low natural cross-pollination rates of 0.03-1.14% and 2.4-3.0% in cultivated and wild soybean, respectively (Pratap *et al.*, 2015). For hybridization or crossing, the anthers are removed from the flowers of a male plant possessing desirable traits (emasculation) and pollen from the anther is transferred to the stigma of flowers of another female plant with favorable characteristics (pollination) (Liu, 1997b).

Since the 1950s, mutation breeding, also known as mutagenesis, has been applied to artificially induce changes in traits of interest within a short period of time. This approach can be helpful when the desired natural genetic variation is limited. As mutations are a naturally occurring phenomena of life, e.g. induced by cosmic and ultraviolet radiation, mutation breeding has been considered as part of conventional plant breeding. In mutation breeding, seeds or plants are treated with physical (e.g. X-rays, gamma-rays, UV-rays or neutrons) or chemical (e.g. ethyl methanesulphonate, ethyleneimine and *N*-nitro-*N*-methylurea) mutagens (Liu, 1997b). In a subsequent step, the segregating populations are subjected to various screening procedures for the trait of interest. Promising homozygous mutants can directly be used for multiplication to generate direct mutant varieties or they can be further modulated by crossing with other genotypes (Maluszynski *et al.*, 1995).

Genetically modified crops have been generated since the 1980s (Fraley *et al.*, 1983). Such biotechnological tools comprise plant tissue cultures, genetic transformation, molecular breeding, and marker-assisted selection (Pratap *et al.*, 2015). Two main

transformation methods for the introduction of DNA are used in soybean, i.e. particle bombardment of proliferative embryogenic cultures and *Agrobacterium*-mediated transformation of cotyledonary nodes (Finer and Larkin, 2008). In 2014, transgenic soybeans were grown on 82% of the global area used for soybean cultivation, making soybean one of the world's biggest commercial successes in transgenic plants (Pratap *et al.*, 2015).

2.2 Phytic acid and lower inositol phosphates

2.2.1 Structure and natural occurrence

Inositol phosphates refer to metabolites with variable phosphate groups attached to the carbocyclic sugar alcohol inositol (Duong *et al.*, 2018). Nine stereoisomeric forms of inositol exist, with *myo*-inositol representing the most abundant form in nature (Murthy, 2006) and the only isomer which is synthesized *de novo* from D-glucose-6-phosphate (Loewus and Murthy, 2000). In its most favorable and stable chair conformation with one axial hydroxyl group at C2 and five equatorial hydroxyl groups, *myo*-inositol can be divided into two non-superimposable mirror image halves between C2 and C5, illustrated by the dashed line in Figure 1a. Phosphorylation at positions C2 or C5 maintains the plane of symmetry and therefore results in achiral *meso* molecules. Phosphorylation at the stereogenic positions C1, C3, C4 or C6 eliminates the plane of symmetry leading to chiral inositol phosphates (Parthasarathy and Eisenberg, 1991).

Agranoff's turtle (Figure 1b) was introduced as a mnemonic to facilitate the numbering and nomenclature of *myo*-inositol and inositol phosphates. The axial 2-hydroxyl represents the turtle's head and the equatorial hydroxyls the limbs and tail. According to the Nomenclature Committee of the International Union of Biochemistry (NC-IUB), the D-numbering system should be applied for *myo*-inositol (IUB Nomenclature Committee, 1989). Therefore, the tutle's right front limb is the 1-D position and numbering proceeds in the counterclockwise direction (Irvine and Schell, 2001; Murthy, 2006).

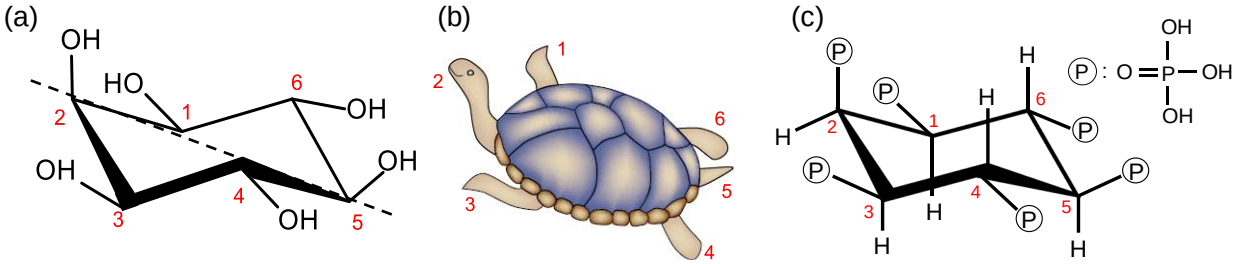

Figure 1. (a) *Myo*-inositol (Ins) in the stable chair conformation with one axial (C2) and five equatorial hydroxyl groups. (b) Agranoff's turtle (Agranoff, 1978), (c) phytic acid (adapted by permission from Copyright Clearance Center's RightsLink®: Springer Nature, Nature Reviews Molecular Cell Biology, Back in the Water: The Return of the Inositol Phosphates, Irvine and Schell, 2001).

Theoretically, 63 *myo*-inositol phosphomonoesters are possible comprising *myo*-inositol mono-, bis-, tris-, tetra*akis*-, penta*akis*- and hexa*akis*phosphate ($InsP_{1\text{-}6}$) (Table 3) (Irvine and Schell, 2001). Besides that, inositol pyrophosphates (PP-$InsP_s$) with pyrophosphate instead of monophosphate moieties attached to the six carbons of inositol, and phosphorylated inositol lipids, so called phosphoinositides, further expand the variety of phosphorylated inositol metabolites found in nature (Livermore *et al.*, 2016).

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*akis*phosphate, $InsP_6$, Figure 1c) and its mixed cationic salts (phytates) are the major storage form of inorganic phosphorus (P) in mature seeds like grains, legumes, oilseeds and nuts, accounting for 65 to 85% of total P (Raboy, 1997) and forming one to several per cent of their dry weight (Lott *et al.*, 2000). The first discovery of phytate dates back to 1855 when Hartig reported small, nonstarch molecules in different plant seeds (Hartig, 1855, 1856; Song *et al.*, 2018). Besides phytate, lower inositol phosphates are also present in seeds, but to a much lower degree (< 15%) (Schlemmer *et al.*, 2009). While inositol phosphates and their salts with monovalent cations are normally water-soluble (Duong *et al.*, 2018), phytate salts with divalent and trivalent cations are usually insoluble (Weaver and Kannan, 2002).

The conformational preferences of inositol phosphates are dependent on pH. In the pH range 0.5-9.0, $InsP_6$ adopts in the sterically stable 1 ax/5 eq (one phosphate in the axial position and five phosphates in the equatorial position) conformation and above pH 9.5 in the sterically hindered 5 ax/1 eq (five phosphates in the axial position and one phosphate in the equatorial position) conformation. Both conformations are in

Table 3. Overview of theoretically possible *myo*-inositol phosphate isomers (shown in D-configuration).

InsP-compound	corresponding enantiomer
Ins(1)P_1	Ins(3)P_1
Ins(2)P_1	
Ins(4)P_1	Ins(6)P_1
Ins(5)P_1	
Ins(1,2)P_2	Ins(2,3)P_2
Ins(1,3)P_2	
Ins(1,4)P_2	Ins(3,6)P_2
Ins(1,5)P_2	Ins(3,5)P_2
Ins(1,6)P_2	Ins(3,4)P_2
Ins(4,5)P_2	Ins(5,6)P_2
Ins(2,5)P_2	
Ins(2,4)P_2	Ins(2,6)P_2
Ins(4,6)P_2	

InsP-compound	corresponding enantiomer
Ins(1,2,3)P_3	
Ins(1,2,4)P_3	Ins(2,3,6)P_3
Ins(2,3,5)P_3	Ins(1,2,5)P_3
Ins(1,2,6)P_3	Ins(2,3,4)P_3
Ins(2,4,6)P_3	
Ins(2,4,5)P_3	Ins(2,5,6)P_3
Ins(1,3,4)P_3	Ins(1,3,6)P_3
Ins(1,5,6)P_3	Ins(3,4,5)P_3
Ins(4,5,6)P_3	
Ins(1,3,5)P_3	
Ins(1,4,6)P_3	Ins(3,4,6)P_3
Ins(1,4,5)P_3	Ins(3,5,6)P_3
Ins(1,2,3,4)P_4*	Ins(1,2,3,6)P_4

InsP-compound	corresponding enantiomer
Ins(1,3,4,5)P_4	Ins(1,3,5,6)P_4
Ins(1,2,5,6)P_4	Ins(2,3,4,5)P_4
Ins(2,4,5,6)P_4	
Ins(1,4,5,6)P_4	Ins(3,4,5,6)P_4
Ins(1,2,4,5)P_4	Ins(2,3,5,6)P_4
Ins(1,2,4,6)P_4	Ins(2,3,4,6)P_4
Ins(1,3,4,6)P_4	
Ins(1,2,3,5)P_4	
Ins(1,2,3,4,5)P_5	Ins(1,2,3,5,6)P_5
Ins(1,2,4,5,6)P_5	Ins(2,3,4,5,6)P_5
Ins(1,3,4,5,6)P_5	
Ins(1,2,3,4,6)P_5	
Ins(1,2,3,4,5,6)P_6	

dynamic equilibrium at pH 9.5. $InsP_5$ adopts the 1 ax/5 eq in the pH range 1.0-9.0; in the pH range 9.5-13.0 both conformations are in dynamic equilibrium. In contrast, the other lower inositol phosphates ($InsP_4$-$InsP_1$) always adopt the 1 ax/5 eq confirmation over the whole pH range (Barrientos and Murthy, 1996).

Phytic acid/phytate and lower inositol phosphate contents in exemplary cereals, legumes, major tree nuts and oilseeds are summarized in Tables 4 and 5. Since 2018, the Global Food and Composition Database for Phytate (PhyFood Comp database – Version 1.0) is available as the first global depository of analytical data on phytate in its different forms determined by different analytical methods with in total 3377 entries both from raw and processed foods (FAO/IZiNCG, 2018).

Table 4. Phytic acid/phytate contents of exemplary cereals, legumes, major tree nuts, and oilseeds (Schlemmer *et al.*, 2009).

Common name	Taxonomic name	Phytic acid/phytate content [mg/g dry matter][a]
Cereals		
Maize	*Zea mays*	7.2 – 22.2
Barley	*Hordeum vulgare*	3.8 – 11.6
Wheat	*Triticum* spp.	3.9 – 13.5
Rice	*Oryza sativa*	0.6 – 10.8
Oats	*Avena sativa*	4.2 – 11.6
Legumes		
Lentils	*Lens culinaris*	2.7 – 15.1
Soybeans	*Glycine max*	10.0 – 22.2
Kidney beans	*Phaseolus vulgaris*	6.1 – 23.8
Chickpeas	*Cicer arietinum*	2.8 – 16.0
Major tree nuts		
Almonds	*Prunus dulcis*	3.5 – 94.2
Cashews	*Anacardium occidentale*	1.9 – 49.8
Hazelnuts	*Corylus avellana*	2.3 – 9.2
Macadamias	*Macadamia integrifolia*	1.5 – 26.2
Oilseeds		
Linseed	*Linum usitatissimum*	21.5 – 36.9
Sesame seed	*Sesmun indicum*	14.4 – 53.6

[a] Depending on the published data

Table 5. Lower inositol phosphate contents in exemplary cereals, legumes and major tree nuts (Duong *et al.*, 2018; Sun and Jaisi, 2018).

		$InsP_5$	$InsP_4$	$InsP_3$	$InsP_2$	$InsP_1$
Common name	Taxonomic name	mg/g dry matter[a]				
Cereals						
Maize[a]	*Zea mays*	0.17 – 1.45	n.d. – 0.35	0.04 – 0.21	–	–
Barley[a]	*Hordeum vulgare*	n.d. – 0.46	n.d.	n.d. – 1.22	–	–
Wheat[a]	*Triticum* spp.	n.d. – 0.29	n.d.	n.d.	–	–
Oats[a]	*Avena sativa*	0.06 – 0.12	n.d.	n.d.	–	–
Legumes						
Lentils[a]	*Lens culinaris*	0.12 – 1.86	0.05 – 0.40	0.13 – 0.17	0.10	0.05
Soybeans[b]	*Glycine max*	1.70 – 2.10	0.30 – 0.50	–	–	–
Kidney beans[a]	*Phaseolus vulgaris*	0.17 – 1.04	0.10	< 0.002	–	–
Chickpeas[a]	*Cicer arietinum*	1.22	0.02 – 0.30	< 0.002 – 0.25	0.10	0.03
Major tree nuts						
Almonds[a]	*Prunus dulcis*	0.23 – 5.80	0.20 – 4.25	0.42 – 0.76	0.24 – 1.29	< 0.001 – 0.39
Cashews[a]	*Anacardium occidentale*	0.75 – 3.83	0.15 – 0.60	0.08 – 0.21	0.02 – 0.07	< 0.001 – 0.05
Hazelnuts[a]	*Corylus avellana*	0.23 – 0.70	0.10 – 0.55	0.004 – 0.21	0.01 – 0.17	0.001 – 0.08
Macadamias[a]	*Macadamia integrifolia*	0.35 – 0.70	0.10	0.01	< 0.001	0.02

[a] Values from Duong *et al.* (2018) and Sun & Jaisi (2018) were converted from µmol/g to mg/g with the following equation (x µmol/g * $M_{inositol\ phosphate}$)/ 1000); M, molar mass; M_{InsP_1} = 260 g/mol; M_{InsP_2} = 340 g/mol; M_{InsP_3} = 420 g/mol; M_{InsP_4} = 500 g/mol; M_{InsP_5} = 580 g/mol; n.d., not detectable and limit of detection was not specified.

Depending on the growing locations and seasons, irrigation conditions, soil types, and application of fertilizers, phytate contents are known to be subject to natural fluctuations (Reddy, 2002). The amount and distribution of phytic acid in seeds can vary considerably between different plant species (Duong *et al.*, 2018). In rice, wheat or barley, phytic acid is mainly accumulated in the aleurone and bran (O'Dell *et al.*, 1972; Raboy, 2003), in maize the embryo is the major storage (O'Dell *et al.*, 1972), and in legume seeds such as common bean, phytic acid is mostly stocked in cotyledons (Ariza-Nieto *et al.*, 2007).

2.2.2 Biosynthesis

The biosynthetic pathway leading to phytic acid in eukaryotic cells is shown in Figure 2, and it can be subdivided into three main steps: (i) supply of the substrates *myo*-inositol (Ins) and *myo*-inositol 3-phosphate (Ins(3)P_1), (ii) conversion of Ins and/or Ins(3)P_1 into phytic acid ($InsP_6$) via the lipid-independent or the lipid-dependent pathway and (iii) transport and storage of phytic acid as globoids inside protein storage vacuoles (Cichy *et al.*, 2009; Raboy, 2003; Sparvoli and Cominelli, 2015). Electron microscopy analysis has suggested that phytic acid is synthesized in the cytosol, transported into the endoplasmic reticulum lumen, and moved in endoplasmic reticulum-derived vesicles to protein storage vacuoles (Greenwood and Bewley, 1984; Otegui *et al.*, 2002).

Substrate supply

The Ins backbone originates from the conversion of D-glucose-6-phosphate to InsP(3)P_1, a highly conserved reaction catalyzed by D-*myo*-inositol 3-phosphate synthase (*MIPS*). Free Ins can be formed via dephosphorylation of InsP(3)P_1 by *myo*-inositol phosphate monophosphatase (*IMP*). The reaction of *IMP* can be reversed by *myo*-inositol kinase (*MIK*) (Raboy, 2003; Sparvoli and Cominelli, 2015).

Conversion of Ins and/or Ins(3)P_1 to phytic acid

The subsequent steps involved in the biosynthesis of phytic acid can be divided into a lipid-independent (Figure 2, left side) and a lipid-dependent pathway (Figure 2, right side). These two routes differ in their early intermediate steps leading to $InsP_3$ (Raboy, 2009).

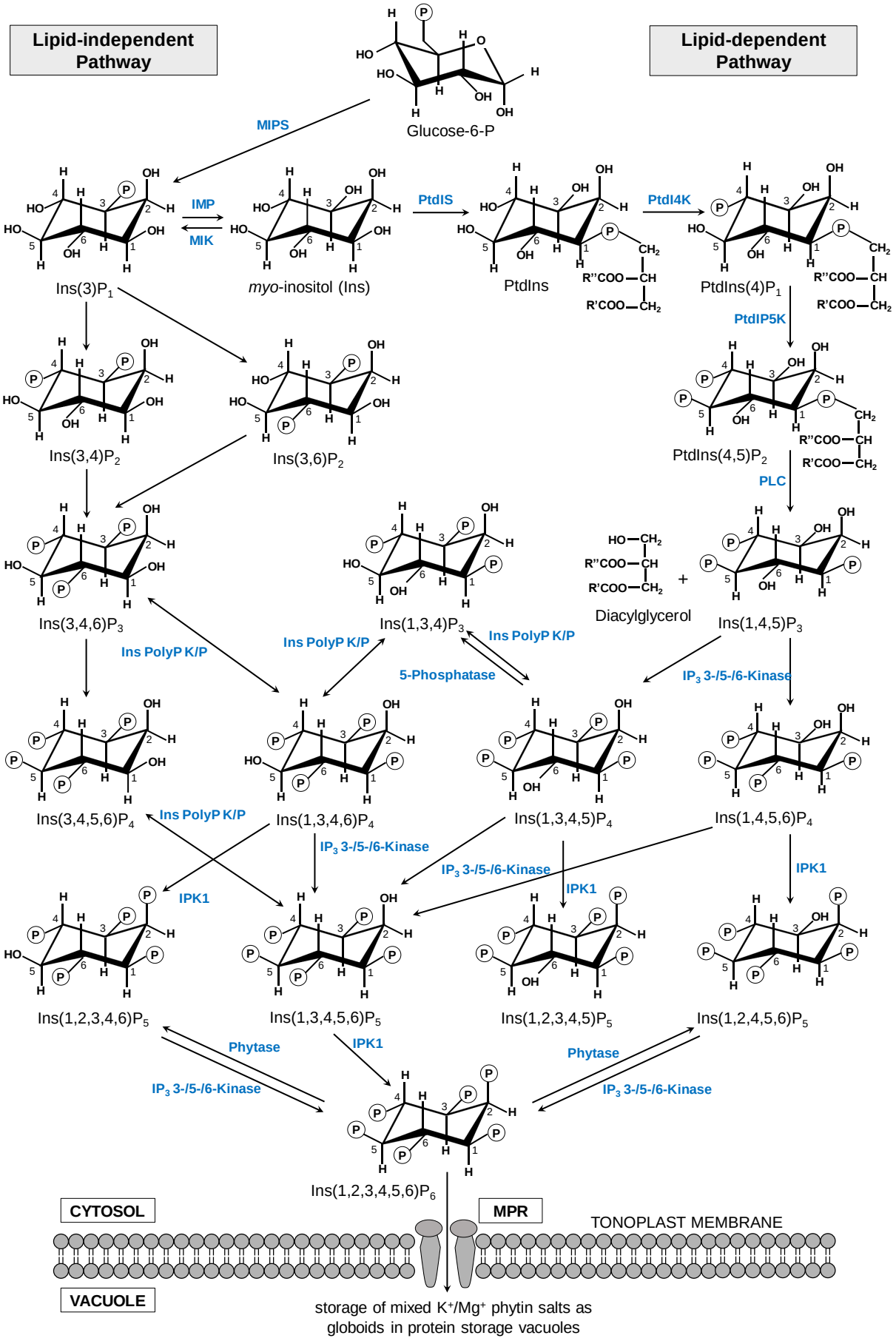

Figure 2. Biosynthetic pathway of phytic acid in eukaryotic cells. (Full caption and abbreviations see next page.)

Figure 2. (continued) The lipid-independent (left side) and the lipid-dependent (right side) pathways for Ins(1,2,3,4,5,6)P_6 synthesis are indicated. The carbons in the inositol ring are numbered according to the D-numbering convention. Enzymes catalyzing critical steps are illustrated in blue. MIPS, D-*myo*-inositol 3-phosphate synthase; IMP, *myo*-inositol phosphate monophosphatase; MIK, *myo*-inositol kinase; PtdIS, phosphatidyl inositol phosphate synthase; PtdI4K, phosphatidyl inositol 4-kinase; PtdIP5K, phosphatidyl inositol 4-phosphate 5-kinase; PLC, phospholipase C; IP_3 3-/5-/6-Kinase, Ins(1,4,5)P_3 3-/5-/6-kinase; Ins PolyP K/P, inositol polyphosphate kinase/ phosphatase; IPK1, inositol 1,3,4,5,6 penta*kis*phosphate 2-kinase; MRP, multidrug-resistance-associated protein ATP-binding cassette (Raboy, 2003, 2009; Sparvoli and Cominelli, 2015).

The lipid-dependent pathway starts with the conversion of Ins into phosphatidyl inositol (PtdIns) by phosphatidyl inositol phosphate synthase (*PtdIS*). Subsequently, phosphatidyl inositol kinases phosphorylate the headgroup of PtdIns to form PtdIns(4,5)P_2, the substrate of phospholipase C (PLC) which releases Ins(1,4,5)P_3. The consecutive phosphorylation steps of Ins(1,4,5)P_3 yielding phytic acid involve different types of Ins polyphosphate kinases. The lipid-independent pathway comprises the consecutive phosphorylation of the Ins ring to form phytic acid, starting with Ins(3)P_1 and involving the action of a number of specific inositol phosphate kinases. The lipid-dependent route is active in all plant tissues (Sparvoli and Cominelli, 2015). Nevertheless, the lipid-independent route is the dominating pathway in the phytic acid biosynthesis of seeds (Raboy, 2009).

Transport and storage of phytic acid

After synthesis, phytic acid is actively transported from the cytosol to the protein storage vacuoles by a multidrug resistance-associated protein (MRP) transporter, belonging to the ATP-binding cassette (ABC) family (Sparvoli and Cominelli, 2014). In protein storage vacuoles, phytic acid is deposited as discrete inclusions, so-called globoids, in the form of mixed phytin salts, primary containing K, Mg and to a lesser extent Ca, Fe and Zn (Raboy, 2009).

2.2.3 Role in plant growth and development

Phosphorus is an essential element of key molecules such as nucleic acid, phospholipids and adenosine triphosphate (ATP), and therefore of fundamental importance for all living organisms (Elser and Bennett, 2011; Tong *et al.*, 2017).

Seed phytate serves as storage for phosphorus, *myo*-inositol and minerals such as the macronutrients potassium, magnesium and calcium and the micronutrients iron, zinc or manganese for germination, early seedling growth, and development (Boehm *et al.*, 2017; Raboy, 1997). Besides that, several genes, enzymes and metabolites of the phytic acid biosynthesis have diverse regulatory roles in physiological and plant developmental processes (Sparvoli and Cominelli, 2015) including signal transduction (Lemtiri-Chlieh *et al.*, 2003), mRNA export (Lee *et al.*, 2015; York *et al.*, 1999), repair of DNA double-strand breaks (Hanakahi *et al.*, 2000), membrane trafficking (Luo *et al.*, 2011; Thole and Nielsen, 2008), basal resistance to plant pathogens (Murphy *et al.*, 2008), osmotic stress signaling (Munnik and Vermeer, 2010), phosphorus homeostasis (Kuo *et al.*, 2014; Stevenson-Paulik *et al.*, 2005), plant photomorphogenesis (Qin *et al.*, 2005) or auxin-regulated embryogenesis (Luo *et al.*, 2011).

2.2.4 Nutritional importance

2.2.4.1 Estimated dietary intake

The daily intake of phytate and other inositol phosphates has been estimated to vary from 180 mg to about 4500 mg on a worldwide basis (Reddy, 2002) and from 300 mg to 2600 mg on the basis of a Western style diet (Schlemmer *et al.*, 2009). In the United Kingdom, the median dietary phytate intakes for children, adolescents, adults, and the elderly population were estimated to be 496 mg, 615 mg, 809 mg, and 629 mg per day, respectively (Amirabdollahian and Ash, 2010). Overall there are considerable differences between the dietary intakes of (i) developing and industrialized countries, (ii) rural and urban areas, (iii) females and males, (iv) infants, children, and adults, and between (v) omnivores and vegetarians (Schlemmer *et al.*, 2009). These huge differences are generally determined by the proportion of plant-derived foods in the diet, the absolute amount of food consumed and the type and extent of applied food processing techniques (Farouk *et al.*, 2017). Several food preparation and processing methods such as milling, soaking, cooking, fermentation or germination are known to reduce the content of phytic acid and its derivatives in the processed materials (Greiner and Konietzny, 2006; Gupta *et al.*, 2013, 2015; Song *et al.*, 2018). For example, cooking reduces the phytate content of soybean by 17% while 72 h of fermentation leads to a reduction of 61% (Marfo *et al.*, 1990). Besides that, the addition of isolated

phytases during food processing (e.g. breadmaking) can additionally contribute to phytate hydrolysis in plant-derived foods (Greiner and Konietzny, 2006).

2.2.4.2 Phytate hydrolysis in the gut, absorption and bioavailability

Subsequent removal of phosphate groups from the inositol ring via hydrolysis decreases the stability of *myo*-inositol phosphate-mineral complexes and increases their solubility eventually leading to a higher bioavailability of essential minerals (Sandberg *et al.*, 1999). Phytate degradation in the human stomach and small intestine can take place by means of phytases of microbial origin produced by the intestinal microflora or by phytases of plant origin (Sandberg, 2002). At the physiological conditions of the gastrointestinal tract, plant phytases are less stable than microbial phytases (Sandberg, 2002). In addition, during food processing steps, plant phytases are mainly inactivated. Therefore, the major phytate hydrolysis takes place in the small intestine by the activity of microbial phytases resulting in an overall low phytate degradation in the gut of humans on a Western-style diet (Schlemmer *et al.*, 2001). In addition, a high calcium intake reduces the intestinal phytic acid degradation, probably by the formation of insoluble calcium-phytate complexes decreasing the accessibility of phytate for enzymatic hydrolysis (Selle *et al.*, 2009; Walker *et al.*, 1948). On the contrary, in humans on diets rich in plant-derived phytases dietary phytate degradations between 36% and 63% have been observed (McCance and Widdowson, 1935). Schlemmer *et al*. (2001) elucidated the pathways of stepwise phytate degradation in different parts of the gut of pigs. The degradation products formed by 6- and 3-phytases from feed or microbial origin comprise among others DL-Ins(1,2,3,4,5)P_5 and DL-Ins(1,2,4,5,6)P_5 as well as inositol phosphates with second messenger activity such as DL-Ins(1,4,5)P_3 and DL-Ins(1,3,4,5)P_4 (Schlemmer *et al.*, 2001).

The contribution of dietary inositol phosphates to the levels found in the human body is controversially discussed, and no carrier has been identified in the gut so far (Duong *et al.*, 2018). The high negative charge density of InsP_6 at physiological pH of about 6-7 was assumed to hamper its transport over cell membranes and therefore gastro-intestinal absorption (Duong *et al.*, 2018; Schlemmer *et al.*, 2009; Vincent *et al.*, 2002). However, results from studies in animals and humans indicate that intracellular inositol phosphate levels in various tissues and biological fluids as well as urinary excretions are related to oral phytic acid intake (Grases *et al.*, 2001b; Grases *et al.*, 2001a).

Experiments in rats showed that soluble radioactive labelled phytic acid applied in drinking water is rapidly absorbed through the stomach and upper small intestine and distributed to various tissues (Sakamoto *et al.*, 1993). Experiments with HeLa cells suggested that cellular uptake of phytate might take place by pinocytosis (Ferry *et al.*, 2002). Receptor-mediated endocytosis has been proposed as an alternative option for the cellular uptake of phytic acid (Vucenik and Shamsuddin, 1994). Wilson *et al.* (2015) did not detect any $InsP_6$ in human plasma or urine by means of a specific titanium dioxide (TiO_2) purification method and therefore concluded that an intestinal phytic acid absorption is unlikely. However, the positive health effects described for phytic acid, such as its anticancer activity (see section 2.2.4.4) strongly support the absorption of either phytic acid or its degradation products in the gut (Schlemmer *et al.*, 2009). Nevertheless, further studies using specific assays are required to reveal possible absorption mechanisms and the bioavailability of dietary inositol phosphates.

2.2.4.3 Antinutritive properties

The presence of phytate in the diet can lead to the formation of complexes between its negatively charged phosphate groups and mineral cations in the gut, resulting in negative effects on the uptake and bioavailability of these minerals (Lopez *et al.*, 2002). Such complexes are soluble under the acidic conditions of the stomach, but they precipitate at neutral pH in the intestine (Schlemmer *et al.*, 2001). Besides the pH value, numerous other factors influence the effect of phytate on the bioavailability of minerals, including valency and size of the minerals, the presence of potential enhancers (e.g. ascorbic acid, *β*-carotene, organic acids) and/or inhibitors (e.g. dietary fiber, polyphenols) in the food matrix as well as the mineral and phytate concentrations and ratios (Schlemmer *et al.*, 2001; Weaver and Kannan, 2002). Titration experiments showed that at pH 7.4, phytic acid and mineral cations can form complexes in the following decreasing order of strength: $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$ (Vohra *et al.*, 1965). Under malnutrition or imbalanced diets low in minerals and essential trace elements but high in phytate, these effects may lead to serious mineral differences from which children, infants and women of childbearing age are primarily affected (Schlemmer *et al.*, 2001). Generally, the mineral binding capacity of inositol phosphates is lowered with decreasing number of phosphate groups per molecule and the formed complexes of such lower inositol phosphates have a higher solubility (Persson *et al.*, 1998). In sucking rats, the zinc and calcium uptake was inhibited by $InsP_6$ and $InsP_5$, but for $InsP_4$ and $InsP_3$ no effects were observable (Lönnerdal *et al.*,

1989). In humans, $InsP_3$ and $InsP_4$ also showed no inhibitory effect on iron absorption, if tested separately. But in mixtures containing inositol phosphates with different phosphorylation stages, lower inositol phosphates may contribute to the inhibitory effect of mineral absorption, possibly by forming mineral complexes between different inositol phosphate isomers (Sandberg *et al.*, 1999).

In addition to minerals, phytate can also form complexes with other positively charged food components such as proteins, carbohydrates and lipids. At a pH value below the isoelectric point of proteins, the cationic groups of basic amino acids (e.g. arginine, histidine or lysine) can form binary insoluble protein-phytate complexes with the phosphoric acid groups of phytate leading to reduced enzymatic activity, protein solubility and digestibility (Kumar *et al.*, 2010). Phytate can hamper the digestion of carbohydrates such as starch by its direct binding, binding with proteins closely associated with starch, chelation of calcium required for the activity of amylase or the association of phytate with other digestive enzymes (Rickard and Thompson, 1997). Furthermore, phytate may form so-called lipophytin complexes with lipids leading to the formation of metallic soaps in the gut lumen and reduced lipid bioavailability (Kumar *et al.*, 2010).

2.2.4.4 Potential health benefits

Besides the described antinutritive properties, phyate has also been shown to have beneficial effects such as antioxidative and anticancer activities. Due to the formation of Fe(III)-phytate complexes, phytate blocks iron-driven hydroxyl radical formation and thereby lipid peroxidation processes (Graf and Eaton, 1990). The same inhibitory effect on hydroxyl radical formation was also shown for Ins(1,2,3,4,6)P_5 and DL-Ins(1,2,3,4,5)P_5, leading to the conclusion that the 1,2,3 (equatorial-axial-equatorial) phosphate grouping contains the essential binding site for iron (Hawkins *et al.*, 1993). Phytate and lower inositol phosphates might also contribute to the antioxidant defense *in vivo* by preventing the formation of reactive oxygen species (ROS) (Burgess and Gao, 2002). Moreover, phytic acid was shown to exhibit anticancer activity against various kinds of cancer e.g. colon, liver, mammary, prostate, lung, liver or blood/bone marrow (Jenab and Thompson, 2002; Nissar *et al.*, 2017; Shamsuddin, 2002; Vucenik and Shamsuddin, 2006). Various mechanisms have been proposed for this anticancer activity including chelating ability, pH reduction, antioxidative functions, interruption of cellular signal transduction, cell cycle inhibition, enhancement of neutral killer (NK) cells activity, and inhibition of angiogenesis (Kumar *et al.*, 2010; Vucenik and

Shamsuddin, 2006). Other studies suggest that phytate may be used to treat hypercalciuria and kidney stones and possesses hypolipidaemic and hypocholesterolemic effects (Shamsuddin, 2002). Its antiplatelet activity has been reported to reduce the risk for coronary heart diseases and ischemic stroke (Vucenik and Shamsuddin, 2006). Moreover, phytic acid may possess beneficial and protective effects against diabetes mellitus by reducing the blood glucose response (Lee *et al.*, 2006).

For several lower inositol phosphates second messenger functions have been described. For example, Ins(1,4,5)P_3 was identified as a second messenger that mediates receptor-induced Ca^{2+} mobilization (Streb *et al.*, 1983). Ins(1,3,4,5)P_4 was found to have important roles in T cell, B cell and neutrophil cell development and function (Sauer and Cooke, 2010), and in NK cell maturation and responsiveness (Sauer *et al.*, 2013). Ins(3,4,5,6)P_4 inhibits Cl^- conductance through the chloride channel ClC-3 *in vivo* (Mitchell *et al.*, 2008). Due to the many roles of ClC-3, Mitchell *et al.* (2008) concluded that Ins(3,4,5,6)P_4 has the potential to regulate neuronal development, tumor cell migration, bone remodeling, apoptosis and inflammatory responses. Furthermore, there are studies indicating that Ins(1,3,4,5,6)P_5 is a specific PtdIns(3,4,5)P_3 competitor and able to block the serine phosphorylation and the kinase activity of the Phosphoinositide 3-kinase/ serine/threonine protein kinase B (PI3K/Akt) signaling pathway resulting in antiangiogenic, proapoptotic and anticancer activities *in vitro* and *in vivo* (Maffucci *et al.*, 2005; Piccolo *et al.*, 2004). Besides that, Ins(1,3,4,5,6)P_5 was shown to sensitize ovarian, lung, and breast cancer cell lines to chemotherapeutic drugs (Piccolo *et al.*, 2004). In this context, Ins(1,3,4,5,6)P_5 and Ins(1,4,5,6)P_4 were even reported to have a higher anticancer activity than $InsP_6$ (Ferry *et al.*, 2002).

2.2.5 Environmental impact

The global formation of phytate by commercially produced crop seeds and fruits has been estimated to reach 51 million tons per year (Lott *et al.*, 2000). Non-ruminant animals such as poultry, swine or fish lack sufficient phytase in their upper digestive tract leading to the excretion of undigested phytate in manure (Erdman, 1979; Wilcox *et al.*, 2000). The application of excess industrially produced mineral fertilizers or manure to farm land increases the phosphorus accumulation in the soil eventually leading to phosphorus pollution of water bodies via drainage, surface runoff, and wind

erosion (Brinch-Pedersen *et al.*, 2002). Eutrophication stimulates algal bloom leading to hypoxic water conditions and eventually the death of aquatic animals and fish (Gupta *et al.*, 2013). Especially under concentrated livestock operations, manure often exceeds the phosphorus requirements of crops to which the manure is applied (Carpenter *et al.*, 1998). Furthermore, the expected increase in livestock production in the future is likely to further exacerbate these environmental issues (Gupta *et al.*, 2013).

To meet the nutritional requirements of non-ruminant animals, inorganic phosphorus is commonly added to animal feed (Wiggins *et al.*, 2018), which additionally contributes to environmental phosphorus pollution. Besides that, supplementation of animal diets with microbial phytase is considered standard in industrial livestock farming (Reis *et al.*, 2018). It was shown to improve the phosphorus bioavailability and to sustainably reduce the phosphorus excretion by up to 50% (Haefner *et al.*, 2005). Transgenetic pigs expressing salivary phytase do not require inorganic phosphate supplementation and excrete up to 75% less phosphorus in manure (Golovan *et al.*, 2001).

2.2.6 *Low phytic acid* crops

Another sustainable and publicly accepted approach to reduce the negative environmental impacts of phytic acid and its antinutritional properties is the generation of *low phytic acid* (*lpa*) crops. Mutations that disrupt the normal biosynthesis and accumulation of phytic acid have been reported in a variety of crops including maize (Raboy *et al.*, 2000; Shi *et al.*, 2007), barley (Larson *et al.*, 1998; Rasmussen and Hatzack, 1998), rice (Ali *et al.*, 2013; Larson *et al.*, 2000; Liu *et al.*, 2007), wheat (Guttieri *et al.*, 2004), soybean (Hitz *et al.*, 2002; Wilcox *et al.*, 2000; Yuan *et al.*, 2007), common bean (Campion *et al.*, 2009), pea (Rehman *et al.*, 2012), and *Arabidopsis thaliana* (Stevenson-Paulik *et al.*, 2005).

Depending on the gene targets in phytic acid biosynthesis or accumulation, *lpa* mutations can be classified into three types as shown in Table 6 (Sparvoli and Cominelli, 2015). In Type 1 *lpa* mutants, the substrate supply pathway is disrupted, i.e. the formation of Ins or Ins(3)P_1. Examples of this type of mutation are the soybean mutants *Gm-lpa*-TW-1 (Yuan *et al.*, 2007) and *Gm-lpa*-TW-1-M (Yuan *et al.*, 2017), carrying a mutated *MIPS1* gene, and the rice mutant *Os-lpa*-XS110-1 with a disruption in *MIK* (Liu *et al.*, 2007). In Type 2 *lpa* mutants, the end of the pathway leading to phytic acid is perturbed, including e.g. the *IPK1* soybean mutant *Gm-lpa*-ZC-2 (Yuan *et al.*,

2012) or the *ITPK4 Arabidopsis thaliana* mutant *atitpk4-1* (Kim and Tai, 2011). Type 3 *lpa* mutations affect the tissue compartmentation, transportation or storage of phytic acid to the vacuole, like in the in the *OsSULTR3;3* rice mutant *Os-lpa*-MH86-1 (Liu *et al.*, 2007; Zhao *et al.*, 2016) or the *MRP* maize mutant *lpa1-7* (Cerino Badone *et al.*, 2012).

Table 6. Classification of *lpa* mutation types depending on the targets in the biosynthesis or accumulation of phytic acid, affected genes and respective metabolic consequences (Sparvoli and Cominelli, 2015).

Mutation type	Disruption in the pathway	Affected gene	Metabolic consequences besides $InsP_6$ reduction
Type 1	Ins and $Ins(3)P_1$ synthesis from glucose 6-P	*MIPS* *MIK* *IMP*	Molar equivalent increase in inorganic P
Type 2	Conversion of Ins and $Ins(3)P_1$ to $InsP_6$	*2-PGK* *IPK2* *ITPK* *IPK1*	Low increase in inorganic P and increased content of lower inositol phosphates
Type 3	Tissue compart-mentation of $InsP_6$ and/or its transport-ation and storage to the vacuole	*MRP* Putative sulfate transporter (*sultr3;3*)	Molar equivalent increase in inorganic P and/or decrease in $InsP_6$ in specific seed tissues

Lpa mutations were in no case spontaneous, emphasizing the fundamental importance of the phytic acid biosynthetic pathway in plants (Sparvoli and Cominelli, 2015). Instead, three general approaches to generate *lpa* mutants can be applied: (i) transgenetic strategies to express recombinant microbial phytases in seeds (Bilyeu *et al.*, 2008; Denbow *et al.*, 1998; Drakakaki *et al.*, 2005; Li *et al.*, 1997), (ii) transgenic lines with disrupted genes involved in phytic acid biosynthesis (Bhati *et al.*, 2016; Nunes *et al.*, 2006; Punjabi *et al.*, 2018; Shi *et al.*, 2007), and (iii) mutation breeding using chemical (e.g. ethyl methanesulfonate or sodium azide) (Hitz *et al.*, 2002; Wilcox *et al.*, 2000) or physical mutagenesis (e.g. X-rays, *γ*-rays, ion beam) (Liu *et al.*, 2007; Yuan *et al.*, 2007). In contrast to genetic engineering, mutation breeding is considered

a conventional breeding technique, publicly accepted, cost-effective and technically easier to implement (Yuan *et al.*, 2007).

Lpa mutants showed reductions in phytic acid contents compared to their respective wild-types ranging from 10% to 90% (Raboy, 2002; Sparvoli and Cominelli, 2015). However, despite the efforts of plant breeders that have identified numerous *lpa* crops, these mutants often show negative effects on seed and plant performance, e.g. reduced yield, compromised germination rate, emergence, stress tolerance, seed filling or grain weight (Raboy, 2007). These negative pleiotropic effects can be explained by the complex role of the phytic acid metabolism in numerous regulatory, physiological, and developmental processes of plants (Sparvoli and Cominelli, 2015) (see Chapter 2.2.3). Indeed, no high-yielding *lpa* cultivars exist to date (Boehm *et al.*, 2017) and cross- and selection-breeding are being applied to overcome these drawbacks (Zhao *et al.*, 2008).

2.2.7 Determination of phytic acid and lower inositol phosphates

The following section gives a brief overview of some of the methods which have been used for the determination of phytic acid and lower inositol phosphates. It should be kept in mind, however, that so far there is no ideal procedure for the separation and measurement of all inositol phosphates taking into consideration their stereoisomers (Duong *et al.*, 2018).

2.2.7.1 Non-specific methods

In the early and mid 1900s, phytic acid was quantified based on the non-specific formation of precipitates with iron(III), first described by Heubner and Stadler in 1914. After hydrochloric acid (HCl) extraction, phytic acid was titrated with ferric chloride ($FeCl_3$) solution against ammonium thiocyanate as indicator and the phytic acid content was quantified based on the quantity of Fe^{3+} (Heubner and Stadler, 1914). However, the definite end point of this titration is difficult to detect and the molar ratios between Fe^{3+} and phytic acid in the complexes are not consistent (Duong *et al.*, 2018). Therefore, the method was adapted by quantifying phytic acid on the basis of the phosphorus content in the precipitates (McCance and Widdowson, 1935).

Harland and Oberleas (1977) eliminated inorganic phosphorus from biological samples by purifying the phytate-containing HCl extract using an AG 1-X8 chloride form anion-exchange resin. After purification, the eluate was digested, inorganic phosphorus was

quantified and the $InsP_6$-equivalent was calculated (Harland and Oberleas, 1977). A modified version of this method was introduced as the official AOAC-method (No. 986.11) for the determination of phytate in food (AOAC, 1990).

The main disadvantages of such non-specific methods are the low sensitivity and the lack of specificity to distinguish between phytic acid, inorganic phosphate and lower inositol phosphates, leading to an overestimation of the phytic acid content in many biological samples (Skoglund and Sandberg, 2002). Furthermore, sugar phosphates, nucleotides, phospholipids and phosphate derivatives of thiamine also contribute to the organic phosphate levels found in foods (Duong *et al.*, 2018). However, similar non-specific precipitation methods are still applied today (Burgos-Luján and Tong, 2015). In general, phytic acid normally represents more than 90% of inositol phosphates in unprocessed foods. Therefore, precipitation methods have been regarded suitable for the determination of phytic acid in raw grains. However, the accurate quantification of phytic acid in processed foods, which typically contain considerable amounts of lower inositol phosphates, requires specific methods such as HPLC or HPIC (Wu *et al.*, 2009).

2.2.7.2 Specific methods

The use of ion-pair octadecyl (C18) reversed-phase high-performance liquid chromatography (HPLC) procedures allowed the study of $InsP_6$, $InsP_5$, $InsP_4$, and $InsP_3$ in food and intestinal contents. After the purification of inositol phosphates on strong anion exchange columns, they were separated using ion-pair HPLC and detected with refractive index detection (Sandberg and Ahderinne, 1986). The drawback of this method is that it does not allow differentiations between isomeric forms of inositol phosphates because gradient elution cannot be used in refractive index detection (Skoglund and Sandberg, 2002). Furthermore, the nucleotide ATP (adenosine triphosphate) was shown to coeluate with $InsP_3$ (Morris and Hill, 1996).

Gas chromatographic (GC) methods have also been used for the analysis of inositol phosphates in food and biological samples, but such methods require the formation of trimethylsilyl derivatives (Hirvonen *et al.*, 1988; March *et al.*, 2001). For example, Heathers *et al.* (1989) dephosphorylated and desalted individual inositol phosphate fractions after HPLC separation, derivatized the resulting *myo*-inositol to hexatrimethylsilyl derivatives and quantified them based on GC analysis.

Nowadays, ion-pair reversed-phase HPLC and anion-exchange HPLC are commonly used to separate inositol phosphates (Duong *et al.*, 2018; Liu *et al.*, 2009). However,

$InsP_3$-$InsP_6$ are better separated with an acidic mobile phase, while alkaline mobile phases were shown to be more powerful for $InsP_1$ and $InsP_2$ (Duong *et al.*, 2018; Schlemmer *et al.*, 2001). *myo*-[^{3}H]inositol or [^{31}P]PO_4^{3-} radiolabeled inositol phosphates can be used to analyze inositol phosphates in biological processes such as cellular signaling by HPLC (Taylor *et al.*, 1990).

Due to their negative charge, inositol phosphates can also be analyzed by capillary electrophoresis (CE) methods, e.g. by capillary zone electrophoresis (CZE) and capillary isotachophoresis (CITP) coupled with mass spectrometry (Buscher *et al.*, 1995), conductivity detection (Blatný *et al.*, 1994) or indirect UV absorbance detection (Buscher *et al.*, 1994). However, these methods only possess limited sensitivity. Furthermore, high-performance thin-layer chromatography (HPTLC) followed by molybdate staining and UV-detection has been applied to separate inositol phosphates (Hatzack and Rasmussen, 1999) as well as inductivity coupled plasma (ICP) methods in combination with atomic emission spectroscopy (AES) (Grases and Llobera, 1996), mass spectrometry (MS) (Muñoz and Valiente, 2003) or tandem mass spectrometry (Zhang *et al.*, 2017).

High performance ion chromatography (HPIC) methods are promising for the discrimination between isomeric forms of inositol phosphates (Skoglund and Sandberg, 2002) and offer good sensitivity and reproducibility allowing the measurement of low concentrations (Wu *et al.*, 2009). The first HPIC method for the in-line determination of $InsP_6$ was developed by Phillippy and Johnston (1985) using a strong anion exchange AS3 column in combination with an HNO_3 gradient, post-column derivatization with $Fe(NO_3)_3$, and UV-detection at 290 nm. Inositol phosphates are not fluorophoric; however, their phosphate groups can be derivatized by Fe^{3+} to form soluble UV-detectable complexes under acidic conditions, allowing their in-line quantitation (Chen and Li, 2003; Oates *et al.;* Phillippy and Johnston, 1985). Other detection methods are fluorescence detection, light scattering detection, conductivity detection, mass spectrometry, refractive index detection (RI) and ^{31}P-nuclear magnetic resonance spectroscopy (NMR) (Schlemmer *et al.*, 2009). NMR allows the identification of stereoisomers that often coeluate during chromatographic separation (Phillippy, 1989). Furthermore, NMR offers high accuracy and specificity and can be applied on intact tissues and cell suspensions, making it especially suitable for studying inositol phosphate metabolism, binding activities, and degradation (Duong *et al.*, 2018). However, the instrumentation is expensive and due to its low sensitivity,

NMR is not suitable for samples with low inositol phosphate concentrations (Skoglund and Sandberg, 2002).

2.3 Plant Metabolomics

2.3.1 Definition

Metabolites are the end products of gene expression which determine the biochemical phenotype of an organism (Sumner *et al.*, 2003). In analogy to the genome, transcriptome, and proteome, the term “metabolome” was introduced in 1998 and is defined as the complete set of all metabolites synthesized by an organism (Oliver *et al.*, 1998). Metabolomics is an unbiased profiling technique belonging to the omics disciplines besides genomics, transcriptomics, and proteomics. Metabolomics aims to comprehensively identify and quantify all intra- and extracellular metabolites with molecular mass lower than 1000 Da (Villas-Bôas *et al.*, 2005). The ultimate goal is to find explanations to biological phenomena under certain conditions. Thus, data analysis and interpretation are essential for any metabolomics experiment (Korman *et al.*, 2012). The metabolome of the plant kingdom has been estimated to comprise 200,000 metabolites (Fiehn, 2001) with up to 20,000 metabolites present in a single plant (Fernie *et al.*, 2004). Due to this high complexity, there is no single method which is able to simultaneously provide a comprehensive snapshot of the metabolome (Villas-Bôas *et al.*, 2005). Instead, the use of pre-fractionation steps and subsequent parallel analyses using a combination of different analytical techniques is applied to overcome this drawback and to obtain as much information as possible (Goodacre *et al.*, 2004; Sumner *et al.*, 2002).

The three major techniques being applied in metabolomics research are (i) targeted analysis, (ii) metabolite fingerprinting, and (iii) metabolite profiling (Putri *et al.*, 2013). Targeted analysis includes the quantitative measurements of the concentrations of a limited number of pre-defined metabolites in a biological sample (Fiehn, 2002). Metabolite fingerprinting is a high-throughput screening tool to discriminate samples of different biological status or origin without the identification or quantification of metabolites (Dunn and Ellis, 2005). In contrast, metabolite profiling involves the identification and quantification (or semiquantification) of specific metabolites belonging to the same class of compounds or to a specific metabolic pathway (Fiehn, 2001; Nielsen, 2007).

2.3.2 Untargeted metabolomics workflow

Metabolomics is a complex interdisciplinary field of research which requires knowledge in life sciences, analytical chemistry, organic chemistry, chemometrics and informatics (Fukusaki and Kobayashi, 2005). The general workflow of untargeted metabolomics studies includes biological study design, sample collection and preparation, raw data acquisition via the analytical platform, data processing and pre-treatment, metabolite identification, statistical analysis for potential biomarker identification, and finally the biological interpretation of the results using pathway and network analysis approaches (Alonso *et al.*, 2015; Hendriks *et al.*, 2011; van den Berg *et al.*, 2006; Wang *et al.*, 2015). These steps are summarized in Figure 3 and will be discussed in detail in the following sections.

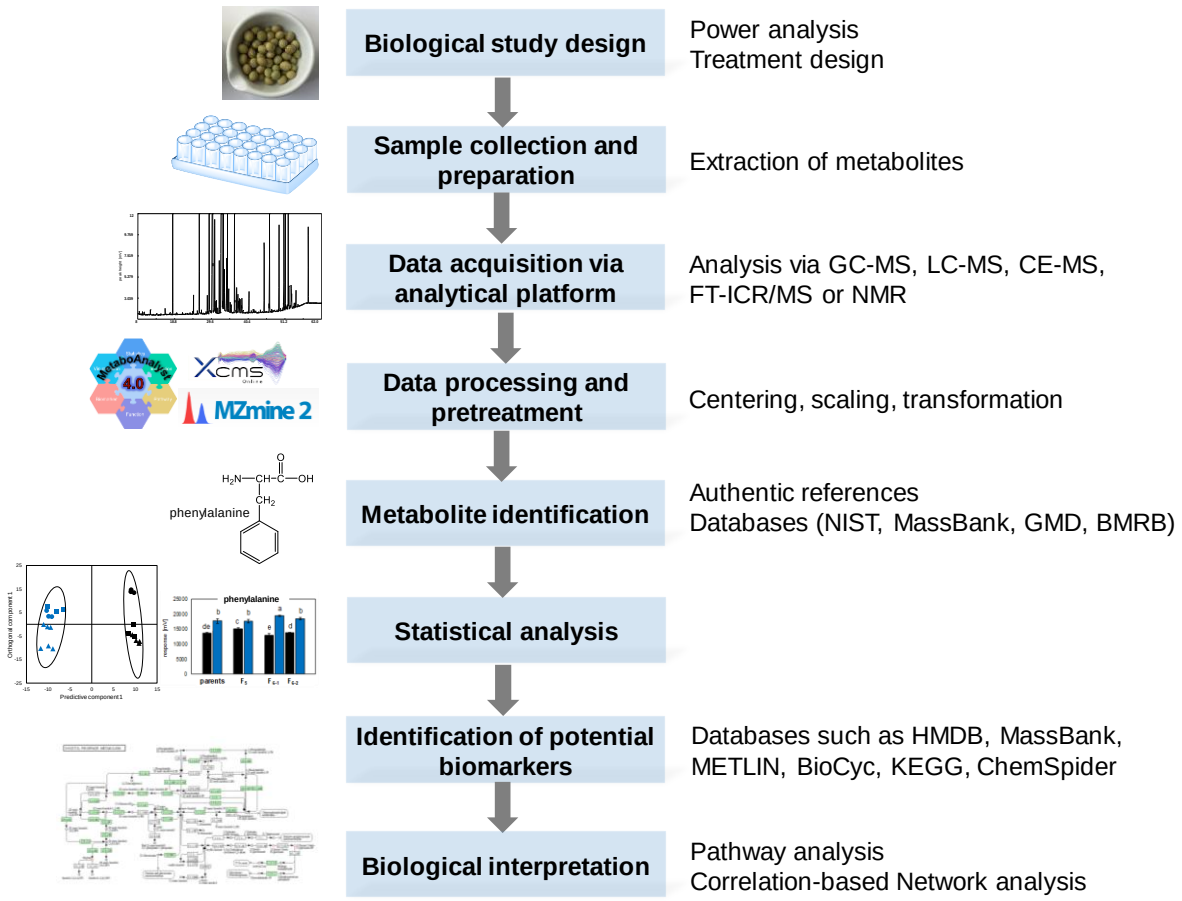

Figure 3. Typical workflow of an untargeted metabolomics study (based on Alonso *et al*., 2015, Hendriks *et al*., 2011, van den Berg *et al*., 2006, Wang *et al.,* 2015).

2.3.3 Analytical techniques

Taking into consideration the complexity of biological samples in metablomics research and the partly low concentrations of individual compounds, extremely high-resolution and sensitive analytical techniques are required (Korman *et al.*, 2012). Mass spectrometry and nuclear magnetic resonance spectroscopy are the two key analytical techniques in metabolomics. MS approaches can be used with prior chromatographic or electrophoretic separation such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) or as direct MS without chromatographic separation, e.g. for metabolite fingerprinting (Dettmer *et al.*, 2007; Halket *et al.*, 2005). An overview of the advantages and disadvantages of the three main metabolomics approaches is given in Table 7.

Mass spectrometers operate by ion formation, separation and subsequent detection of ions based on their mass-to-charge (*m/z*) ratio (Dunn and Ellis, 2005). Molecules can be ionized by electron ionization (EI), a hard ionization technique where molecules are broken into several fragments. Alternatively, soft ionization, which does not introduce significant fragmentation of the molecular ions, can be used, e.g. chemical ionization (CI), atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) (Korman *et al.*, 2012; Smedsgaard, 2007). The most frequently used ionization methods in metabolomics are EI (for GC-MS) and ESI (for HPLC-MS) (Gowda and Djukovic, 2014). Depending on the applied type of ionization technique, positive, negative or both types of ions can be generated.

The most common single-configuration mass analyzers are linear quadrupole (Q), ion trap, time of flight (TOF), and Fourier transform ion cyclotron resonance (FT-ICR); with increasing price, resolution, and sensitivity, respectively, in this order. The combination of different mass analyzers, e.g. Q-TOF, offers mass determination with a high accuracy of 1-5 ppm and sensitivity in the nanomolar or even picomolar concentration range (Korman *et al.*, 2012).

The most commonly used stationary phases of capillary GC columns are dimethyl polysiloxane, 5% diphenyl/95% dimethyl polysiloxane, and trifluoropropylmethyl polysiloxane (Yang *et al.*, 2013). A requirement for GC-MS analysis is volatility and thermal stability of the analytes. Large molecules or smaller molecules with polar groups tend to have a lower volatility, leading to extremely long retention times or non-

Table 7. Comparison of the three main metabolomics approaches (modified from Wishart (2008)).

Technology	Advantages	Disadvantages
GC-MS	- robust and mature technology - relatively inexpensive - good sensitivity - detects most organic and some inorganic molecules - quantitative (with standard calibration) - modest sample size required - large body of software and databases for metabolite identification - excellent separation reproducibility	- samples not recoverable - non-volatiles need derivatization - not suitable for heat-labile compounds - requires extensive sample preparation - longer measurement time - identification of unknown compounds is difficult
LC-MS	- good sensitivity - detects most organic and some inorganic molecules - minimal sample size required - direct injection without separation possible - flexible technology - has potential for detecting largest proportion of the metabolome	- samples not recoverable - limited quantification - relatively expensive instrumentation - limited body of software and databases for metabolite identification - less robust instrumentation than NMR or GC-MS - worse separation resolution and reproducibility compared to GC - identification of unknown compounds is difficult - longer measurement time
NMR	- quantitative - non-destructive - no separation required - no derivatization required - detection of all organic compound classes - short measurement time (2-3 min per sample) - robust and mature technology - large body of software and databases for metabolite identification - straightforward structural identification of unknown compounds - compatible with liquids and solids	- relatively low sensitivity - expensive instrumentation - detection limited to protonated compounds - no detection of salts and inorganic ions - relatively large sample volume (0.5 mL) required

elution of the compounds, decomposition or asymmetric chromatographic peaks when analyzed by means of gas chromatography (Drozd, 1975). Therefore, polar metabolites normally require derivatization steps at their functional groups to increase thermal stability and volatility for GC analysis, to improve chromatographic separation and peak shape, and to reduce the reactivity of the compounds and the possibility of intermolecular associations (Drozd, 1975; Lisec *et al.*, 2006). Alkylation, acylation or silylation can be used for active hydrogens in functional groups like –COOH, –OH, -NH, or -SH (Dettmer *et al.*, 2007). In plant metabolite profiling, *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) and methoxyamine hydrochloride are the most frequently used derivatization reagents. However, it should be kept in mind that derivatization of metabolites can result in more than one peak for a specific compound of interest due to partial silylation or isomerization in case of methoxyaminated compounds like sugars (Lisec *et al.*, 2006). In addition, some derivatives can decompose by the action of heat, moisture or light. Therefore, derivatization should always be carried out shortly before GC analysis (Drozd, 1975).

In contrast to GC, liquid chromatography can also be used to analyze thermolabile and high molecular weight molecules including hydrophilic, lipophilic, neutral, acidic, and basic compounds (Hopfgartner and Varesio, 2013). An advantage of liquid chromatography is that extracted samples can directly be analyzed without the requirement for derivatization steps (DeVos *et al.*, 2007). In more than 80% of the separations, reversed-phase (RP) chromatography is used, especially octadecyl (C18) columns; for nonpolar molecules, normal-phase (NP) chromatography can be employed (Glauser *et al.*, 2013). Highly polar metabolites are typically not retainable on classical reversed-phase stationary phases (Dettmer *et al.*, 2007). In such cases, hydrophilic interaction liquid chromatography (HILIC) can be employed (Tolstikov and Fiehn, 2002).

In NMR spectroscopy, strong magnetic fields and radio frequency (RF) pulses are applied to the nuclei of atoms. In atoms with odd atomic (e.g. ^{1}H) or mass numbers (e.g. ^{13}C), such magnetic fields will result in a nuclear spin. The absorption of RF energy conveys the nuclei from a low-energy to a high-energy spin state. During the relaxation process, the subsequent radiation emission is measured (Dunn and Ellis, 2005). The spin and magnetic moment properties of the nuclei in a molecule are dependent on their chemical environment (Smedsgaard, 2007). Thereby, NMR can provide comprehensive structural information, including stereochemical properties.

Furthermore, NMR can easily be used for quantitation because the signal intensity is directly proportional to the molar concentration (Kim *et al.*, 2011).

The most important nuclei in biological samples are the hydrogen isotope ^{1}H (99.98% natural abundance), the phosphor isotope ^{31}P (100% abundance), and the carbon isotope ^{13}C (1.11% abundance) (Smedsgaard, 2007). In metabolomics, one-dimensional (1D) ^{1}H NMR is the most popular NMR approach (Markley *et al.*, 2017), allowing the measurement of hundreds or even thousands of samples in a short period of time (Bingol and Brüschweiler, 2014). In addition, higher-dimensional NMR such as 2D NMR experiments can provide more detailed and resolved information on both known and unknown metabolites at the expense of longer measurement times. Besides liquid samples, semi-solid samples like tissues can also be analyzed by means of NMR spectroscopy (Bingol and Brüschweiler, 2014). Even though NMR is less sensitive than MS, it allows a rapid and high-throughput analysis for which no or at least only minimal sample preparation steps are required (Kim *et al.*, 2011).

2.3.4 Data pre-processing and pre-treatment

Data pre-processing and pre-treatment are essential to improve the biological interpretability of metabolomics data. After data acquisition, pre-processing steps are used to transform raw instrumental data to clean data (van den Berg *et al.*, 2006). Such pre-processing methods include deconvolution (separation of overlapping signals into individual chemical peaks), peak picking, alignment (correcting shifts in retention times between different runs), baseline correction, and missing value imputation (Karaman, 2017). For the latter, different methods have been proposed to maintain the integrity of the dataset, e.g. replacing missing values by means or medians of the metabolite level across different samples or by means of nearest neighbors (Steuer *et al.*, 2007).

Prior to multivariate statistical approaches such as PCA or OPLS-DA, additional pre-treatment steps of the clean data are necessary to focus on the biologically relevant information in the data and properly interpret the results (Goodacre *et al.*, 2007; Karaman, 2017). Data pre-treatment methods include normalization, centering, scaling, and transformation (Karaman, 2017; Liland, 2011). For example, internal or external standards can be used for data normalization to remove unwanted systematic variation between samples and to make observations more directly comparable (Hendriks *et al.*, 2011; Karaman, 2017). By centering, differences in the offset between high and low abundant metabolites are adjusted to focus on the relevant variation

between samples (van den Berg *et al.*, 2006). By the application of scaling methods such as autoscaling, pareto scaling, range scaling, and vast (variable stability) scaling, it can be avoided that highly abundant metabolites mask low abundant metabolites, which might be even more important from a biological point of view (Karaman, 2017; van den Berg *et al.*, 2006). Log transformation, generalized log (glog) transformation or power transformation can be applied to approximate the normal distribution and to correct heteroscedastic noise (Karaman, 2017). Which data pre-treatment is most suitable for a respective dataset depends on several factors, e.g. the biological information to be obtained and the chosen data analysis (van den Berg *et al.*, 2006). Currently, there is no general agreement on which transformation and scaling methods are most suitable for metabolomics datasets. Instead, different types of transformation and scaling methods as well as their combinations should be tested to find the most suitable approach to approximate the normal distribution for the respective dataset (Xia and Wishart, 2016).

Several software tools for metabolomics data processing are available, including commercial software, such as Sieve (Thermo Fisher Scientific, Waltham, MA, USA), MassHunter Mass Profiler Software (Agilent Technologies, Santa Clara, CA, USA), or metAlign (PlanResearch International, Wageningen, The Netherlands) (Katajamaa and Oresic, 2007), and free software, e.g. Chrom*pare* (Frenzel *et al.*, 2003), MZmine (Katajamaa *et al.*, 2006), XCMS (Smith *et al.*, 2006), or HiRes (Zhao *et al.*, 2006).

2.3.5 Statistical analysis

Metabolomics experiments result in complex and huge amounts of raw data. Appropriate statistical tools, including multivariate and univariate methods, are necessary to extract relevant information and to better understand and interpret the underlying structure of the data.

2.3.5.1 Multivariate analysis

Unsupervised methods

Principal component analysis (PCA) is one of the oldest (Hotelling, 1933; Pearson, 1901) and most widely used multivariate processing approaches in Metabolomics to identify chemical differences between samples. For PCA, the dimensionality of data is reduced by eliminating the higher principle components while maintaining as much as possible of the variation in the dataset. This is achieved by a linear transformation to a

new coordinate system, the so-called principle components (PCs), so that the greatest variance of the data is found on the first axis (PC1) and the second largest variance on the second axis (PC2) (Hansen, 2007). PCA data are visualized by means of two types of plots, the score plot and the loading plot. The score plot shows the distribution trend of data points and the loading plot reflects which metabolites provoke this discrimination trend (Wu and Wang, 2015).

Besides PCA, cluster analyses, comprising hierarchical cluster analysis (HCA), K-means clustering, or self-organizing maps (SOMs) serve as unsupervised approaches in metabolomics. These methods group and visualize samples depending on their intrinsic similarities using distance metrics such as Euclidean distance, Manhattan distance, or correlation (Sumner *et al.*, 2003).

Supervised methods

While the unsupervised PCA algorithm can be used to obtain an unbiased dimensionality reduction, it will only reveal a group structure as long as the between-group variation is sufficiently more pronounced than the within-group variation (Worley and Powers, 2016). Alternatively, unsupervised methods including partial least square analysis (PLS), partial least square discriminant analysis (PLS-DA), orthogonal partial least square discriminant analysis (OPLS-DA), or soft independent modeling of class analogy (SIMCA) can be used (Bylesjö *et al.*, 2006). PLS-DA is one of the most extensively applied data analysis techniques in metabolomics (Barker and Rayens, 2003). For this approach, samples are classified into pre-defined groups, e.g. wild-type and mutant, which are used to maximize the group separation according to class belonging (Goodpaster and Kennedy, 2011; Xia and Wishart, 2016).

In contrast to PLS-DA, the OPLS-DA model is rotated so that the class variation is found on the first predictive component and the within class variation not related to the response (noise) on the y-orthogonal component to simplify the interpretation of the model (Wiklund *et al.*, 2008). The so-called S-plot (Wiklund *et al.*, 2008) and variable importance in projection (VIP) values (Pérez-Enciso and Tenenhaus, 2003) can be used to assist in the identification of metabolites contributing to the discrimination between groups seen in the OPLS-DA score plot.

Supervised methods like PLS and PLS-DA force the separation between experimental groups in score plots thereby normally resulting in a better class-separation compared to PCA. Thereby, such supervised methods tend to easily overfit models and

overestimate their predictability, i.e. models can just by accident result in a good classification of the groups (Triba *et al.*, 2015; Westerhuis *et al.*, 2008). Therefore, the reliability and robustness of such models requires rigorous validation, e.g. by permutation tests (random permutation of group membership) or by a K-fold cross-validation (splitting the dataset K-times into a training and a test set) (Triba *et al.*, 2015).

2.3.5.2 Univariate analysis

Parametric and nonparametric procedures are available to determine whether a metabolite shows statistically significant differences between the investigated groups. The decision which univariate test is the most suitable depends on several statistical properties of the data which have to be tested in advance, including normality (e.g. Shapiro-Wilk or Kolmogorov-Smirnov test), homogeneity of variances (e.g. Levene's or Bartlett's test), and independence. Two events are statistically independent if the occurrence of one event does not affect the probability of occurrence of the other (Vinaixa *et al.*, 2012). For normally distributed metabolites with homogeneity of variances, parametric tests such as unpaired or paired *t*-test (two group comparisons) as well as analysis of variance (ANOVA) followed by multiple pairwise comparisons with post hoc tests such as Tukey's honestly significant difference (HSD) or Fisher's least significant difference (LSD) test are conducted The respective nonparametric alternatives for two unpaired group comparisons are Mann-Whitney *U*-test (unpaired groups), Wilcoxon signed-rank test (paired groups), and Kruskal-Wallis test for multiple comparisons (Jones, 2002; Vinaixa *et al.*, 2012). Alternatively, non-normally distributed data can be transformed to normal or near to normal data using for example logarithmic, square root, or arcsin transformation (Conover and Iman, 1981).

In metabolomics studies normally hundreds of metabolites are determined which all need to be tested for significance (Saccenti *et al.*, 2014). However, the more hypotheses are tested on the same set of data, the higher the probability of wrongly rejecting the null hypothesis by chance, leading to substantial increases in the number of false positives (Type I error). This phenomenon is also known as inflation of the α-level (Abdi, 2007). For a single test, an α-level of 0.05 means that the probability of making a Type I error is equal to $\alpha = 0.05$ and the probability of not making a Type I error is consequently equal to $1-\alpha = 0.95$. For multiple tests, the probability of at least one Type I error increases, e.g. for $c = 3$ independent tests the probability is 0.143. In general terms, the probability to find at least one Type I error in c independent tests on

the same data set is equal to $1-(1-\alpha)^c$ (Abdi, 2007). Therefore, in case of multiple hypothesis-testing, the significant level needs to be protected against an increasing probability of getting false positive results by adjusting the significance level to the number of tests performed, e.g. by Bonferroni correction or by controlling the false discovery rate (ration of the number of false positives to the number of positives) (Benjamini and Hochberg, 2000; Vinaixa *et al.*, 2012). Even though Bonferroni is the most conservative correction, which strictly controls Type I error at expenses of Type II errors (false negative), it is recommended as the best option in metabolomics (Broadhurst and Kell, 2006).

2.3.6 Applications

Over the last decade, numerous metabolomics-based studies have been performed in various fields of plant science, e.g. to investigate the responses of plants to external stresses, to support functional genomics, to analyze metabolic pathways of primary and secondary plant compounds, to follow developmental changes, or to characterize and assess the safety of genetically modified (GM) crops (Frank and Engel, 2013; Glauser *et al.*, 2013). In the following, several exemplary applications of plant metabolomics research are presented.

2.3.6.1 Biotic and abiotic stress responses

During their development, plants are often exposed to different environmental perturbations, including biotic and abiotic stresses. Metabolomics can help to better understand the genetic and biochemical mechanisms underlying the plant's responses to such stresses, and to elucidate metabolic biomarker and potential targets for genetic engineering of stress tolerant plants (Hong *et al.*, 2016).

Metabolomics has been used to investigate the metabolic effects of cold and freezing stress (Welti *et al.*, 2002), heavy metal stress (Le Lay *et al.*, 2006), salt treatment (Kim *et al.*, 2007), light stress (Bino *et al.*, 2005), UV-B radiation (Kaling *et al.*, 2015), flooding (Komatsu *et al.*, 2014), sulfur deficiency (Nikiforova *et al.*, 2005), phosphorus stress (Hernández *et al.*, 2007), oxidative stress (Baxter *et al.*, 2007), as well as drought stress (Lanzinger *et al.*, 2015; Wenzel *et al.*, 2015). Besides that, plants' metabolic responses to the combination of different stresses (Hung and Wang, 2018; Rizhsky *et al.*, 2004; Sun *et al.*, 2015) and to plant pathogens (Hung and Wang, 2018; Jones *et al.*, 2011; Sana *et al.*, 2010) have been investigated.

2.3.6.2 Plant functional genomics

In functional genomics, large datasets originating from genomics, transcriptomics, proteomics, and metabolomics are combined to decode the function of specific genes in the plant genome (Saito and Matsuda, 2010). Even though mRNA profiling is still the primary tool used in plant functional genomics, metabolite profiling approaches are becoming increasingly important (Trethewey and Krotzky, 2007). In the field of functional genomics, metabolite profiling can also be used as an additional tool for the elucidation and characterization of an unknown mutation event, e.g. induced by mutation breeding (Frank, 2009). An important model organism in plants functional genomics is *Arabidopsis thaliana,* for which the whole genome sequence has been determined (Arabidopsis Genome Initiative, 2000). Several *Arabidopsis* knockout mutants and their respective wild-types have been compared by means of metabolite profiling to elucidate the annotation of genes involved in the glucosinolate, pyridine alkaloid, triterpenoid, flavonoid, and sterol metabolism (Lisec *et al.*, 2006). In addition, metabolomics has been applied to determine the location of quantitative trait loci (QTL) in genome-wide analyses, e.g. for *Arabidopsis* (Keurentjes *et al.*, 2006), tomato (Schauer *et al.*, 2006), and rice (Matsuda *et al.*, 2012).

2.3.6.3 Developmental changes

It is well known that the metabolites levels in plants vary depending on the growth stage. Such developmental changes resulting in synthesis, transport, accumulation, or degradation of specific metabolites finally influence the compositional and nutritional quality of foods (Harrigan *et al.*, 2007). Metabolomics approaches have been used to investigate these dynamic changes during growth and development, e.g. in tobacco leaves (Zhang *et al.*, 2018), walnut kernels (Rao *et al.*, 2016), strawberry (Zhang *et al.*, 2011), and hot pepper (Jang *et al.*, 2015). Besides that, the time-dependent changes during germination of rice seeds (Shu *et al.*, 2008), sprouting of mung bean (Na Jom *et al.*, 2011), and malting of barley (Frank *et al.*, 2011; Heuberger *et al.*, 2014) as well as the biochemical changes during pre-harvest fruit development, ripening, and postharvest shelf-life in tomato fruits (Oms-Oliu *et al.*, 2011) have been characterized. The ultimate goal of such approaches is to gain a deeper insight into the complexity of compositional changes during growth and development of plants and to identify metabolite biomarkers which might enable the prediction and monitoring of the physiological and developmental status (Trethewey and Krotzky, 2007).

2.3.6.4 Phytochemical diversity

Cultivation and breeding of crops have a long history, resulting in large germplasm collections with a wide array of genetic diversity. Metabolomics has the potential to explore the phytochemical diversity across different plant varieties (Kusano *et al.*, 2015). Such variability has been investigated, e.g. in diverse varieties of potato (Dobson *et al.*, 2010; Oertel *et al.*, 2017), cassava (Drapal *et al.*, 2019), rice (Kusano and Saito, 2012), and cranberry (Brown *et al.*, 2012) as well as in green tea cultivars (Fujimura *et al.*, 2011). Information on the chemical characteristics of specific varieties may help breeders to select genotypes with desirable nutritional traits for the generation of new, healthy and high quality cultivars. Besides that, metabolomics experiments have also been used to compare foods grown under different farming practices. Differences between conventional versus organic farming have been investigated e.g. in maize (Röhlig and Engel, 2010), beetroot (Kazimierczak *et al.*, 2014), tomato and pepper (Novotná *et al.*, 2012).

2.3.6.5 Characterization and safety assessment of genetically modified crops

A pragmatic tool in the safety assessment of genetically modified crops is based on the so-called concept of substantial equivalence: if a GM food is found to be substantially equivalent to its isogenic non-transgenic counterpart, the GM food can be regarded as safe as the conventional food (OECD, 1993). Such assessments need to take into consideration both intended und unintended effects that may result from the genetic modification (FAO/WHO, 2016). Therefore, the unbiased metabolite profiling approach can be considered as an additional tool for the safety assessment of GM crops. Such comparisons between GM and conventional crops have been performed for example in potato (Catchpole *et al.*, 2005; Roessner *et al.*, 2001), wheat (Baker *et al.*, 2006), maize (Barros *et al.*, 2010; Frank *et al.*, 2012b), and soybean (García-Villalba *et al.*, 2008). In most cases, the majority of differences observed between GM and non-GM foods were related to natural variability including environmental factors such as growing season and growing location rather than the genetic modification itself (Barros *et al.*, 2010; Frank *et al.*, 2012b).

3 RESULTS

3.1 Publication I

Goßner, S.; Yuan, F.; Zhou, C.; Tan, Y.; Shu, Q.; Engel, K. H.
Impact of Cross-Breeding of *Low Phytic Acid MIPS1* and *IPK1* Soybean (*Glycine max* L. Merr.) Mutants on their Contents of Inositol Phosphate Isomers.
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Phytic acid ($InsP_6$), the major storage form of phosphorus in soybean (*Glycine max* (L.) Merr.) seeds, is considered as an antinutrient. Therefore, various *low phytic acid* (*lpa*) soybean mutants have been developed via mutation breeding. Cross-breeding is often used to generate new cultivars with improved agronomic or nutritional properties. However, the knowledge on the consequences of such cross-breeding steps of *lpa* soybean mutants on the contents of $InsP_6$ and lower inositol phosphate isomers ($InsP_2$-$InsP_5$) in the obtained progenies is limited. Therefore, homozygous *lpa* mutant, homozygous double *lpa* mutant and homozygous wild-type (WT) progenies were generated by crossing the *lpa* soybean mutants *MIPS1* and *IPK1* with commercial WT cultivars or among themselves. The seeds were subjected to a high pressure ion chromatography (HPIC)-based approach for the determination of $InsP_2$-$InsP_6$. The *lpa* trait of the *MIPS1* mutant was not changed by crossing with a WT cultivar; *lpa* progenies showed $InsP_6$ reductions of about 44% compared to the respective WT progenies. The $InsP_6$ reduction in *IPK1* progenies varied between 43% and 71% and was dependent on the WT crossing parent. In addition, the *IPK1* progenies accumulated considerable amounts of the lower inositol phosphate isomers $Ins(1,5,6)P_3$/$Ins(3,4,5)P_3$, $Ins(4,5,6)P_3$, $Ins(1,4,5,6)P_4$/$Ins(3,4,5,6)P_4$, and $Ins(1,3,4,5,6)P_5$ in comparison to the progenitor *lpa* mutant. The generation of homozygous *lpa* double mutants, simultaneously carrying the *MIPS1* and *IPK1* mutation target, offered the potential to drastically reduce $InsP_6$ up to 87%, with only moderate accumulations of $InsP_3$-$InsP_5$ isomers. The study demonstrated that cross-breeding can be applied to alter the concentrations of both $InsP_6$ and $InsP_3$-$InsP_5$ in *lpa* soybean seeds and thereby increasing their nutritional quality.

Candidate's contribution:

Implementation of a high pressure ion chromatography (HPIC) method for the analysis of phytic acid and lower inositol phosphate isomers in soybean; identification and quantitation of phytic acid and lower inositol phosphate isomers in all investigated soybean samples; validation of the HPIC method; statistical assessment and interpretation of the elaborated data; writing and revision of the complete manuscript including all Figures and Tables as well as the Supporting Information.

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Impact of Cross-Breeding of *Low Phytic Acid MIPS1* and *IPK1* Soybean (*Glycine max* L. Merr.) Mutants on Their Contents of Inositol Phosphate Isomers

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Supporting Information

ABSTRACT: The knowledge on consequences of cross-breeding of induced *low phytic acid* (*lpa*) soybean (*Glycine max* L. Merr.) mutants on the contents of phytic acid ($InsP_6$) and lower inositol phosphate isomers ($InsP_2$−$InsP_5$) in the resulting progenies is limited. Therefore, *MIPS1* and *IPK1 lpa* soybean mutants were crossed with wild-type (WT) cultivars or among themselves to generate homozygous *lpa* and WT progenies and double *lpa* mutants. The *lpa* trait of the *MIPS1* mutant was not altered by cross-breeding with a WT cultivar; *lpa* progenies had $InsP_6$ reductions of about 44% compared to WT progenies. *IPK1* progenies showed pronounced accumulations of specific $InsP_3$−$InsP_5$ isomers (up to 12.4 mg/g) compared to the progenitor *lpa* mutant (4.7 mg/g); the extent of $InsP_6$ reduction (43−71%) was depending on the WT crossing parent. Double mutants exhibited the most pronounced $InsP_6$ reductions (up to 87%), accompanied by moderate accumulations of $InsP_3$−$InsP_5$ (2.5 mg/g). Cross-breeding offers the potential to modulate the amounts of both $InsP_6$ and $InsP_3$−$InsP_5$ contents in *lpa* soybean mutants and thus to improve their nutritional quality.

KEYWORDS: *low phytic acid mutant, soybean (Glycine max L. Merr.), cross-breeding, inositol phosphates, MIPS1, IPK1*

INTRODUCTION

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, $InsP_6$) is the major storage form of phosphorus (P) in crops accounting for ∼75% of total P in mature seeds.[1] In soybean (*Glycine max* L. Merr.), contents between 1.0 and 4.6% have been described.[2,3] Humans and nonruminant animals cannot digest phytate because they lack sufficient phytase in their digestive tract. The undigested phytate is excreted in animal waste leading to phosphorus pollution and eutrophication of waterways.[4] Furthermore, $InsP_6$ can chelate cationic metal micronutrients like iron and zinc and may interact with proteins making them nutritionally unavailable.[5]

To reduce these negative effects of $InsP_6$, various *low phytic acid* (*lpa*) crops, e.g., soybean, rice, maize, barley, common bean, and wheat have been developed. Genetic engineering[6] as well as mutation breeding, e.g., through chemical mutagenesis[7−9] or γ-irradiation,[10] resulted in phytic acid contents 50−95% lower than in the respective wild-types (WT).[5] The *lpa* soybean mutant lines *Gm-lpa*-TW-1 (TW-*lpa*), *Gm-lpa*-TW-1-M (TW-1-M-*lpa*), and *Gm-lpa*-ZC-2 (ZC-*lpa*) have been generated from the WT cultivars Taiwan 75 and ZC-3, respectively, via 150 Gy ^{60}Co γ-irradiation.[11−13] Molecular characterization revealed that TW-*lpa* and TW-1-M-*lpa* resulted from a 2 bp deletion in the D-*myo*-inositol 3-phosphate synthase gene 1 (*MIPS1*),[11,13] responsible for the conversion of D-glucose 6-phosphate to 1D-*myo*-inositol 3-phosphate ($Ins(3)P_1$), the initial step in the biosynthesis of phytic acid. ZC-*lpa* resulted from a G → A mutation in chromosome 14 of the inositol 1,3,4,5,6-penta*kis*phosphate 2-kinase (*GmIPK1*),[12] which converts $InsP_5$ into phytic acid.

Frank et al. (2009) compared the contents of phytic acid and lower inositol phosphates ($InsP_3$−$InsP_5$) in the wild-types Taiwan 75 and ZC-3 and in the corresponding *lpa* mutants TW-*lpa* and ZC-*lpa*.[14,15] In TW-*lpa*, the $InsP_6$ reduction of 53% was accompanied by a molar equivalent increase in inorganic phosphorus (Pi), but there were no changes in the contents of lower inositol phosphates. In contrast, an accumulation of lower inositol phosphates besides the intended $InsP_6$ reduction is a characteristic feature of ZC-*lpa*.[15] Vincent et al. (2015) demonstrated that $InsP_4$ and $InsP_5$ detected in ZC-*lpa* correspond to the isomers $Ins(1,3,4,5,6)P_5$ and $Ins(1,4,5,6)P_4$/$Ins(3,4,5,6)P_4$.[16] Only few other data on inositol phosphate isomers in soybean seeds are available.[17−19] In particular, direct comparisons of the profiles of inositol

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phosphate isomers of *lpa* soybean mutants and their wild-types are missing.

Cross-breeding is often applied to generate new cultivars showing, for example, improved agronomic properties.[20] For soybeans, data on the contents of phytic acid and lower inositol phosphate isomers in the progenies have been provided for a cross of ZC-*lpa*, which carries an *IPK1* mutation on chromosome 14, with an independent *IPK1* mutant carrying the same mutation on chromosome 6.[16] The resulting double mutant progenies showed drastic phytic acid-P reductions between 79 and 88% accompanied by significant Ins(1,3,4,5,6)P_5 and Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 accumulations.[16] However, the effects of crossing an *lpa* soybean mutant with either a commercial WT cultivar or with another *lpa* soybean mutant carrying a different mutation target on the contents of inositol phosphate isomers in the resulting progenies have not been investigated.

Therefore, the objectives of this study were (i) to perform a comparative assessment of the amounts of $InsP_6$ and lower inositol phosphate isomers in the *lpa* mutants TW-*lpa* (*MIPS1*), TW-1-M-*lpa* (*MIPS1*), and ZC-*lpa* (*IPK1*) and their corresponding WTs Taiwan 75 and ZC-3, (ii) to investigate whether cross-breeding of the *MIPS1* and *IPK1* mutants with commercial WT cultivars leads to changes of the intended $InsP_6$ reductions and the occurrence of lower inositol phosphate isomers in the resulting progenies, and (iii) to determine the extent of phytic acid reduction and the lower inositol phosphate contents in double mutants simultaneously carrying the two mutation targets *IPK1* and *MIPS1*.

■ MATERIALS AND METHODS

Chemicals. Phytic acid dodecasodium salt (≥97%) and iron(III) nitrate nonahydrate (≥99.95%) were obtained from Sigma-Aldrich (Steinheim, Germany), hydrochloric acid (0.5 M, analytic grade) and magnesium sulfate heptahydrate (≥99.5%) from Merck (Darmstadt, Germany), perchloric acid eluent concentrate (0.33 M, HPLC grade) from Bernd Kraft (Duisburg, Germany), deionized water (LC-MS grade) from VWR International (Darmstadt, Germany), and sodium oxalate (≥99.5%) from Alfa Aesar (Karlsruhe, Germany). D-*myo*-Inositol-4,5-diphosphate, D-*myo*-inositol-1,5,6-triphosphate, D-*myo*-inositol-2,4,5-triphosphate, D-*myo*-inositol-1,3,4,5,6-pentaphosphate, and D-*myo*-inositol-1,2,4,5,6-pentaphosphate (all ≥98%) were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA) and D-*myo*-inositol-1,4,5,6-tetraphosphate (≥98%) from Carbosynth Limited (Compton, UK) as sodium salts.

Sample Materials. The *lpa* soybean mutants *Gm-lpa*-ZC-2 (ZC-*lpa*) and *Gm-lpa*-TW-1 (TW-*lpa*) have previously been generated from the WT cultivars Zhechun No. 3 (ZC-3) and Taiwan 75, respectively, via 150 Gy ^{60}Co γ-irradiation.[12,13] In addition, *Gm-lpa*-TW-1-M (TW-1-M-*lpa*), a natural mutant of TW-*lpa* carrying the same *MIPS1* mutation site but showing a significantly higher rate of field emergence compared to TW-*lpa*,[11] was analyzed. Zhexiandou No. 4 (ZXD) and Cu, commercial soybean cultivars showing good germination rates (≥85%) and dry matter yields of 2−2.25 tons/ha, and ZC-3, the progenitor of ZC-*lpa*, were used as wild-type crossing parents.

In total, progenies were obtained from four crosses, i.e., TW-1-M-*lpa* × ZXD, ZC-*lpa* × ZC-3, ZC-*lpa* × Cu, and ZC-*lpa* × TW-1-M-*lpa*. Because of poor seed viability, no progenies had been obtained from the cross Taiwan 75 × TW-*lpa*. Seeds were harvested and bulked from individual plants grown in neighboring plots in the same fields of the experimental farm of Zhejiang Academy of Agricultural Sciences, Hangzhou, and in experimental fields in Haining, Zhejiang province, China, in spring (April-July) and autumn (August-October) up to generation F_8 (Table S1). After freeze-drying (Alpha 1−4 LSC, Christ, Osterode, Germany) for 24 h and grinding with a cyclone mill equipped with a 500 μm sieve (Foss, Rellingen, Germany), the resulting flour was freeze-dried again for 48 h and stored at −18 °C until high pressure ion chromatography (HPIC) analysis.

Genotyping of Progenies. Segregating plants of every generation were genotyped by competitive amplification of differentially melting amplicons analysis (CADMA-HRM) using DNA extracts of leaf tissues as previously described.[21] These extracts were used for asymmetric PCRs with competitive primers to distinguish homozygous *lpa* mutants, homozygous WTs, and heterozygous progenies by high-resolution melting (HRM) curves[21] (Figures S3−S5).

Preparation of an In-house Reference Standard Solution (InsP-Mix). A reference standard solution of inositol phopsphate isomers was prepared by HCl-catalyzed hydrolysis of phytic acid.[22] Briefly, 1 g of phytic acid and 100 mL of 0.5 M HCl were heated under reflux in a 250 mL glass flask at 95−100 °C for 68 h. The resulting stock solution was stored at 4 °C; for subsequent uses, it was diluted with deionized water to a final concentration of 5%. The analysis of this mixture via HPIC resulted in 25 peaks (Figure S1A) representing 32 *myo*-inositol phosphate isomers (6 $InsP_2$, 12 $InsP_3$, 9 $InsP_4$, 4 $InsP_5$, and $InsP_6$), without separation of enantiomers[22] (Table S2). In the following, all possible enantiomers are indicated in the D-configuration.

Analysis of Phytic Acid and Lower Inositol Phosphate Isomers. For the extraction and analysis of phytic acid and lower inositol phosphate isomers, a procedure previously described for dried distiller grains[22] was adapted to the soybean matrix. For *IPK1* mutants, 50 mg of freeze-dried soybean flour was weighed into a 50 mL polystyrene centrifuge tube, and 7.5 mL of 0.5 M HCl was added. The mixture was vortexed, and inositol phosphates were extracted under sonication (15 min). The tube was centrifuged at 4500*g* and 20 °C for 30 min. Three milliliters of the supernatant were diluted with 3 mL of deionized water, the mixture was filled into Vivaspin 6 centrifugal concentrators (30,000 MWCO, PES membrane, Sartorius, Göttingen, Germany) and centrifuged at 4000*g* and 20 °C for 1 h to remove potentially interfering matrix species like proteins. The filtrate was passed through an unconditioned Dionex OnGuard II Ag−H cartridge (2.5 cm^3, Thermo Fisher Scientific, Waltham, MA, USA) to remove excess chloride ions. The first three turbid drops were discarded, and the following 1 mL was collected and passed through a 0.2 μm PES syringe filter disk.

WT and *MIPS1* mutant soybean seeds were expected to contain only minor amounts of lower inositol phosphates;[15] therefore, 400 mg of freeze-dried soybean flour was subjected to the HCl-extraction. The remaining procedure was the same as described for the *IPK1* mutants, except for a prolongation of the ultrafiltration time to 2 h. All samples were worked-up in duplicate, stored at 4 °C and immediately analyzed by HPIC (injection volume: 100 μL).

Analysis of inositol phosphates was performed on a Dionex ICS-5000$^+$ HPIC system equipped with a CarboPac PA100 guard column (4 × 50 mm) and an analytical column (4 × 250 mm) kept at 30 °C (Thermo Fisher Scientific, Waltham, MA, USA). Gradient elution was performed by two mobile phases, i.e., (A) deionized water and (B) 0.5 M HCl, at a flow rate of 1.0 mL/min as follows: −0.1−0 min, 5% B; 0−8 min, 5−10% B; 8−25 min, 10−35% B; 25−40 min, 35−100% B; 40−46 min, 100% B; 46−46.2 min, 100−5% B. An auxiliary pump was used for postcolumn derivatization with 0.1% $Fe(NO_3)_3$ in 0.33 M perchloric acid at a flow rate of 0.4 mL/min. Inositol phosphates were detected with ultraviolet absorbance at 290 nm by an UltiMate 3000 Variable Wavelength Detector.

Identification of Inositol Phosphate Isomers. Identifications of inositol phosphate isomers were based on comparisons of the retention times with those of commercially available reference compounds. Otherwise, tentative identifications were performed by assigning the respective peak in the in-house reference standard solution on the basis of the HPIC data available from the literature.[22,23]

Quantitation of Inositol Phosphate Isomers. External standard calibration curves of D-*myo*-inositol-4,5-diphosphate, D-*myo*-inositol-2,4,5-triphosphate, D-*myo*-inositol-1,4,5,6-tetraphosphate, D-

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Table 1. Inositol Phosphate Isomers (mg/g Dry Matter)[a,b] in the Wild-type Taiwan 75 and the Corresponding *MIPS1* Mutants TW-*lpa* and TW-1-M-*lpa*

	WT	*lpa* mutant 1		*lpa* mutant 2	
inositol phosphate isomer (peak no.)[c]	Taiwan 75 (Hangzhou, spring 2015)	TW-*lpa* (Hangzhou, spring 2014)	TW-*lpa* (Hangzhou, spring 2015)	TW-1-M-*lpa* (Hangzhou, spring 2014)	TW-1-M-*lpa* (Hangzhou, autumn 2016)
$InsP_2$					
$Ins(1,3)P_2$ (**1**)	–[d]	<0.13[e]	<0.13[e]	<0.13[e]	<0.13[e]
$Ins(1,2)P_2$ (**2**)	<0.13[e]	–	–	–	–
$InsP_3$					
$Ins(1,2,3)P_3$ (**9**)	<0.14[e]	–	–	–	–
$Ins(1,2,6)P_3$ (**9**)					
$Ins(1,4,6)P_3$ (**9**)					
$InsP_4$					
$Ins(1,2,3,4)P_4$ (**15**)	<0.05[e]	–	–	–	–
$Ins(1,3,4,6)P_4$ (**15**					
$Ins(1,2,5,6)P_4$ (**18**)	<0.05[e]	–	–	–	–
$Ins(1,4,5,6)P_4$ (**20**)	–	–	–	–	0.06 ± 0.00
$InsP_5$					
$Ins(1,2,3,4,6)P_5$ (**21**)	<0.09[e]	<0.09[e]	<0.09[e]	<0.09[e]	<0.09[e]
$Ins(1,2,3,4,5)P_5$ (**22**)	0.38 ± 0.02 a	0.17 ± 0.01 bc	0.18 ± 0.01 b	0.15 ± 0.00 c	0.15 ± 0.00 c
$Ins(1,2,4,5,6)P_5$ (**23**)	0.28 ± 0.01 a	0.20 ± 0.01 b	0.16 ± 0.00 d	0.20 ± 0.00 b	0.18 ± 0.00 c
$Ins(1,3,4,5,6)P_5$ (**24**)	<0.09[e]	–	<0.09[e]	<0.09[e]	0.17 ± 0.00
$InsP_6$					
$Ins(1,2,3,4,5,6)P_6$ (**25**)	13.30 ± 0.14 a	9.63 ± 0.06 c	8.69 ± 0.41 d	9.90 ± 0.06 bc	10.34 ± 0.14 b
$InsP_2$–$InsP_6$ (sum)	13.95 ± 0.15 a	10.00 ± 0.06 c	9.03 ± 0.42 d	10.25 ± 0.05c	10.91 ± 0.13 b

[a]Values represent means ± standard deviations resulting from duplicate analysis of two aliquots of freeze-dried flour. [b]Different letters indicate statistically significant differences (ANOVA with Tukey HSD test, $p < 0.05$) in specific inositol phosphate isomers between different soybean cultivars and/or growing years. [c]Only one of the possible enantiomers (Table S2) is listed. [d]Content below limit of detection (i.e., 1.1 μg of $InsP_2$, 1.2 μg of $InsP_3$, 0.5 μg of $InsP_4$, 0.8 μg of $InsP_5$, and 0.7 μg of $InsP_6$ per mL injection solution); determined on the basis of 100 μL HPIC injection volume. [e]Content below limit of quantification (mg/g dry matter), determined based on aqueous calibration solutions without consideration of the soybean matrix.

myo-inositol-1,3,4,5,6-pentaphosphate, and phytic acid were used in the calibration range of 0.7–500 μg/mL for the quantitation of inositol phosphate isomers ($R^2 > 0.9959$, Table S4).

Validation of the Methods. Repeatability, reproducibility, and recovery rate were determined in triplicate for each of the 25 peaks, representing in total 32 different $InsP_2$–$InsP_6$ isomers. To this end, 50 mg and 400 mg of commercially available soybean flour, respectively, were spiked with 880 μL of undiluted InsP-Mix, subjected to the sample extraction procedures, and analyzed by HPIC. Mean repeatability was 2.6% RSD (relative standard deviation) and 1.1% RSD for samples of 50 and 400 mg, respectively. Mean reproducibilities (three repeated sample work-ups by the same operator on three consecutive days) were 4.1% RSD (50 mg) and 3.1% RSD (400 mg), and mean recoveries were 102% (50 mg) and 96% (400 mg) (Table S5).

Limits of detection (LODs) and limits of quantification (LOQs) were calculated as 3.3 and 10 times the standard deviation of the response of a blank divided through the slope of the corresponding calibration curves,[24] respectively (Table S4).

Statistical Analysis. Values were expressed as mean ± standard deviation (SD) resulting from the duplicate analysis of two aliquots of freeze-dried flour. ANOVA analysis with Tukey HSD post hoc test ($p < 0.05$) was performed by XLSTAT (version 2018.3.50851, Addinsoft, Paris, France).

RESULTS

Comparison of Inositol Phosphate Isomers in Wild-types and Their *lpa* Mutants. *MIPS1 Mutants TW-lpa and TW-1-M-lpa.* In the wild-type Taiwan 75, phytic acid and the $InsP_5$ isomers $Ins(1,2,3,4,5)P_5$/$Ins(1,2,3,5,6)P_5$ (**22**) and $Ins(1,2,4,5,6)P_5$/$Ins(2,3,4,5,6)P_5$ (**23**) could be quantitated. Six further $InsP_2$–$InsP_5$ isomers were detected, but their contents were below the LOQs. The mutation of the *MIPS1* gene led to significant phytic acid reductions of about 31% and 24% in the mutants TW-*lpa* and TW-1-M-*lpa*, respectively. Different growing years/seasons led to only slight variations in the contents of inositol phosphate isomers of the two *lpa* mutants (Table 1).

IPK1 Mutant ZC-lpa. Similar to Taiwan 75, only the $InsP_5$ isomers (**22**) and (**23**) could be quantitated in ZC-3 (Table 2). In both growing years, ZC-*lpa* showed a mean phytic acid reduction of 39% accompanied by a consistent detection of four further inositol phosphate isomers, with particularly high concentrations of $Ins(1,4,5,6)P_4$/$Ins(3,4,5,6)P_4$ (**20**) and $Ins(1,3,4,5,6)P_5$ (**24**). Whereas the influence of the two different growing years was negligible for phytic acid, the sum of $InsP_4$–$InsP_5$ isomers was higher in autumn 2016 (5.4 mg/g dry matter) than in spring 2014 (3.9 mg/g).

Cross-Breeding of the *MIPS1* Mutant TW-1-M-*lpa* with a WT Cultivar. To investigate the stability of the *MIPS1 lpa* phenotype, the *lpa* mutant TW-1-M-*lpa* was crossed with the commercial WT cultivar ZXD. The contents of phytic acid and lower inositol phosphates in the crossing parents and in the homozygous WT and homozygous *MIPS1* mutant progenies from generations F_3 and F_5 are presented in Table 3. The WT crossing parent ZXD showed an inositol phosphate pattern comparable to the WT cultivar Taiwan 75 (Table 1), except for a higher phytic acid content. After the cross-breeding step, homozygous *lpa* mutant progenies of generations F_3 and F_5 consistently showed statistically significant reductions in phytic acid contents compared to both the respective homozygous WT progenies and to the original WT Taiwan 75. Depending on the growing year, the reduction of

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Table 2. Inositol Phosphate Isomers (mg/g Dry Matter)[a,b] in the Wild-type ZC-3 and the Corresponding *IPK1* Mutant ZC-*lpa*

	WT	*lpa* mutant	
inositol phosphate isomer (peak no.)[c]	ZC-3 (Hangzhou, spring 2014)	ZC-*lpa* (Hangzhou, spring 2014)	ZC-*lpa* (Hangzhou, autumn 2016)
$InsP_3$			
$Ins(1,2,3)P_3$ (**9**)	<0.14[e]	–	–
$Ins(1,2,6)P_3$ (**9**)			
$Ins(1,4,6)P_3$ (**9**)			
$Ins(1,5,6)P_3$ (**12**)	–[d]	<1.08[e]	<1.08[e]
$Ins(4,5,6)P_3$ (**13**)	–	<1.08[e]	<1.08[e]
$InsP_4$			
$Ins(1,2,5,6)P_4$ (**18**)	<0.05[e]	–	–
$Ins(1,4,5,6)P_4$ (**20**)	–	2.22 ± 0.12	3.28 ± 0.09
$InsP_5$			
$Ins(1,2,3,4,5)P_5$ (**22**)	0.29 ± 0.01	<0.73[e]	<0.73[e]
$Ins(1,2,4,5,6)P_5$ (**23**)	0.36 ± 0.02	<0.73[e]	<0.73[e]
$Ins(1,3,4,5,6)P_5$ (**24**)	–	1.70 ± 0.04	2.12 ± 0.05
$InsP_6$			
$Ins(1,2,3,4,5,6)P_6$ (**25**)	19.20 ± 0.12 a	11.50 ± 0.31 b	11.87 ± 0.14 b
$InsP_2$–$InsP_6$ (sum)	19.85 ± 0.14 a	15.42 ± 0.39 c	17.27 ± 0.12 b

[a]Values represent means ± standard deviations resulting from duplicate analysis of two aliquots of freeze-dried flour. [b]Different letters indicate statistically significant differences (ANOVA with Tukey HSD test, $p < 0.05$) in specific inositol phosphate isomers between different soybean cultivars and/or growing years. [c]Only one of the possible enantiomers (Table S2) is listed. [d]Content below limit of detection (i.e., 1.1 μg of $InsP_2$, 1.2 μg of $InsP_3$, 0.5 μg of $InsP_4$, 0.8 μg of $InsP_5$, and 0.7 μg of $InsP_6$ per mL injection solution); determined on the basis of 100 μL HPIC injection volume. [e]Content below limit of quantification (mg/g dry matter), determined based on aqueous calibration solutions without consideration of the soybean matrix.

phytic acid in homozygous *lpa* mutant progenies compared to the homozygous wild-type progenies varied between 39 and 49%. The phytic acid contents of the homozygous *lpa* progenies of generations F_3 and F_5 were in the same range as in the crossing parent TW-1-M-*lpa*, and the concentrations of the $InsP_5$ isomers (**22**) and (**23**) were slightly higher than those in the *lpa* mutant crossing parent. However, they remained in the same order of magnitude.

Cross-Breeding of the *IPK1* Mutant ZC-*lpa* with WT Cultivars. Table 4 summarizes the contents of phytic acid and lower inositol phosphates in the crossing parents ZC-*lpa* and the commercial soybean cultivar Cu as well as in their homozygous WT and *lpa* mutant progenies of generation F_5. The inositol phosphate profile of Cu was comparable to those of Taiwan 75 and ZC-3, except for the additionally quantitated $InsP_3$ isomer (**9**) and the additionally detected $InsP_4$ isomers (**15**), (**18**), and (**20**). The phytic acid content of the homozygous *lpa* mutant progeny was not affected by the cross-breeding step. However, the *lpa* mutant progeny showed an almost 2.5 times higher concentration (9.7 mg $InsP_3$–$InsP_5$/g) of the lower inositol phosphates (**12**), (**20**), (**22**), (**23**), and (**24**) than the *lpa* mutant crossing parent ZC-*lpa* (3.9 mg $InsP_3$–$InsP_5$/g). Consequently, the total inositol phosphate content in the homozygous *IPK1* mutant progeny was not only higher than in ZC-*lpa* but even higher than in the original WT ZC-3 and the crossing parent Cu.

After crossing ZC-*lpa* with its WT progenitor ZC-3, the intended $InsP_6$ reduction was more pronounced in the homozygous *IPK1* mutant progenies and was further increased with progressing generations. Whereas in the parental generation the phytic acid reduction was about 40%, the *lpa* mutant progeny of generation F_6 exhibited a 69% lower phytic acid content than the corresponding WT progeny (Table 4). This increasing reduction of phytic acid over generations was accompanied by an increased accumulation of lower inositol phosphate isomers. In the homozygous *lpa* progenies of generation F_6, the amounts of the $InsP_5$ isomer (**24**) and the $InsP_4$ isomer (**20**) were significantly higher than in ZC-*lpa*, and in addition, the $InsP_3$ isomers (**12**) and (**13**) could be quantitated. The total $InsP_2$–$InsP_6$ contents were significantly higher in the *lpa* mutant progenies compared to the *lpa* mutant crossing parent ZC-*lpa*, but they were still lower than those in ZC-3 and the homozygous WT progenies (Table 4).

Generation of a Double *lpa* Mutant by Crossing of ZC-*lpa* (*IPK1*) and TW-1-M-*lpa* (*MIPS1*). Inositol phosphate isomers in homozygous double WT and homozygous double *lpa* mutant (*IPK1*/*MIPS1*) progenies of generations F_3 to F_8 resulting from the crossing of the *IPK1* mutant ZC-*lpa* with the *MIPS1* mutant TW-1-M-*lpa* are shown in Table 5. In generation F_3, the single *MIPS1* mutation led to a phytic acid decrease of about 56% compared to the homozygous double WT progeny, whereas the double mutation resulted in a more pronounced reduction of 77%. The content of phytic acid in the homozygous double *lpa* mutant progeny was drastically reduced compared to the starting *lpa* crossing parents. The by far lowest phytic acid content was found in the homozygous double *lpa* mutant of generation F_8, corresponding to 20 and 23%, respectively, of the *lpa* crossing parents ZC-*lpa* and TW-1-M-*lpa*. The double mutants accumulated the lower inositol phosphate isomers $Ins(1,4,5,6)P_4$/$Ins(3,4,5,6)P_4$ (**20**) and $Ins(1,3,4,5,6)P_5$ (**24**), but to a lesser extent than ZC-*lpa* or *lpa* mutant progenies only carrying the *IPK1* mutation target (Table 4). Isomers that were only present at lower concentrations in the *IPK1* mutant ZC-*lpa*, i.e., $InsP_3$ (**12**) and $InsP_5$ (**22**, **23**), were not detectable in double mutant progenies. The different growing seasons and locations also had an influence on the contents of lower inositol phosphates and phytic acid both for the double WT and the double *lpa* mutant progenies.

DISCUSSION

***MIPS1* and *IPK1* Mutants.** For Taiwan 75 and its *MIPS1* mutant TW-*lpa* the results demonstrate that the previously reported low amount of $InsP_5$[15] corresponds to the isomers $Ins(1,2,3,4,5)P_5$/$Ins(1,2,3,5,6)P_5$ (**22**) and $Ins(1,2,4,5,6)P_5$/$Ins(2,3,4,5,6)P_5$ (**23**). These $InsP_5$ isomers have also been identified as quantitatively dominating in other soybean seeds.[18,19] Owing to the disruption of the early inositol phosphate intermediate pathway by the *MIPS1* (D-*myo*-inositol 3-phosphate synthase 1) mutation (Figure 1), both the biosynthesis of lower inositol phosphate intermediates and the formation of phytic acid are disturbed. As a result, their concentrations are further reduced in TW-*lpa* (Table 1). TW-1-M-*lpa* has been reported as a natural mutant of the TW-*lpa* line with the same mutation site (*MIPS1*) and the same phytic

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Table 3. Inositol Phosphate Isomers (mg/g Dry Matter)[a,b] in the Crossing Parents TW-1-M-*lpa* (*MIPS1*) and ZXD and Their Homozygous Wild-type (WT) and Homozygous *lpa* Mutant (*lpa*) Progenies of Generations F_3 and F_5

	crossing parents (Hangzhou, spring 2014)		progenies F_3 (Hangzhou, spring 2014)		progenies F_5 (Hangzhou, spring 2015)	
inositol phosphate isomer (peak no.)[c]	ZXD	TW-1-M-*lpa*	WT	*lpa*	WT	*lpa*
$InsP_2$						
$Ins(1,3)P_2$ (**1**)	–[d]	<0.13[e]	<0.13[e]	<0.13[e]	–	<0.13[e]
$Ins(1,2)P_2$ (**2**)	<0.13[e]	–	–	–	–	–
$InsP_4$						
$Ins(1,2,3,4)P_4$ (**15**) $Ins(1,3,4,6)P_4$ (**15**)	–	–	–	–	<0.05[e]	–
$Ins(1,2,5,6)P_4$ (**18**)	–	–	<0.05[e]	–	<0.05[e]	–
$InsP_5$						
$Ins(1,2,3,4,6)P_5$ (**21**)	–	<0.09[e]	0.16 ± 0.00	<0.09[e]	<0.09[e]	<0.09[e]
$Ins(1,2,3,4,5)P_5$ (**22**)	0.30 ± 0.00 c	0.15 ± 0.00 e	0.44 ± 0.02 a	0.21 ± 0.00 d	0.32 ± 0.01 b	0.30 ± 0.00 c
$Ins(1,2,4,5,6)P_5$ (**23**)	0.27 ± 0.00 c	0.20 ± 0.00 e	0.71 ± 0.01 a	0.31 ± 0.01 b	0.30 ± 0.00 b	0.25 ± 0.01 d
$Ins(1,3,4,5,6)P_5$ (**24**)	–	<0.09[e]	<0.09[e]	–	–	–
$InsP_6$						
$Ins(1,2,3,4,5,6)P_6$ (**26**)	16.06 ± 0.07 c	9.90 ± 0.06 e	16.96 ± 0.27 b	8.61 ± 0.03 f	17.58 ± 0.07 a	10.64 ± 0.03 d
$InsP_2$–$InsP_6$ (sum)	16.63 ± 0.08 b	10.25 ± 0.05 d	18.26 ± 0.29 a	9.13 ± 0.04 e	18.20 ± 0.08 a	11.18 ± 0.02 c

[a]Values represent means ± standard deviations resulting from duplicate analysis of two aliquots of freeze-dried flour. [b]Different letters indicate statistically significant differences (ANOVA with Tukey HSD test, $p < 0.05$) in specific inositol phosphate isomers between the two crossing parents and the corresponding progenies of different generations. [c]Only one of the possible enantiomers (Table S2) is listed. [d]Content below limit of detection (i.e., 1.1 μg of $InsP_2$, 1.2 μg of $InsP_3$, 0.5 μg of $InsP_4$, 0.8 μg of $InsP_5$, and 0.7 μg of $InsP_6$ per mL injection solution), determined on the basis of 100 μL HPIC injection volume. [e]Content below limit of quantification (mg/g dry matter), determined in aqueous calibration solutions without consideration of the soybean matrix.

acid content, but higher seed field emergence.[11] This study showed that not only the phytic acid contents of TW-1-M-*lpa* and TW-*lpa* were comparable, but also their patterns and contents of lower inositol phosphate isomers.

For the *IPK1* mutant ZC-*lpa*, the findings are in line with results from Frank et al. (2009),[14,15] who reported a phytic acid reduction of 46% in ZC-*lpa* and an accumulation of $InsP_3$, $InsP_4$, and $InsP_5$. The previously described presence of the lower inositol phosphate isomers $Ins(1,4,5,6)P_4$/$Ins(3,4,5,6)P_4$ (**20**) and $Ins(1,3,4,5,6)P_5$ (**24**)[16] could be confirmed. In addition, the $InsP_3$ isomers $Ins(1,5,6)P_3$/$Ins(3,4,5)P_3$ (**12**) and $Ins(4,5,6)P_3$ (**13**) as well as the $InsP_5$ isomers $Ins(1,2,3,4,5)P_5$/$Ins(1,2,3,5,6)P_5$ (**22**) and $Ins(1,2,4,5,6)P_5$/$Ins(2,3,4,5,6)P_5$ (**23**) were described in ZC-*lpa* for the first time (Table 2). The $InsP_3$ isomer (**12**) has been reported in corn diets for broilers with and without phytase supplementation.[25]

Cross-Breeding of the *MIPS1* and *IPK1* Mutants. Cross-breeding of the *MIPS1* mutant TW-1-M-*lpa* with the commercial WT cultivar ZXD did not change the phytic acid range in homozygous *lpa* mutant progenies compared to TW-1-M-*lpa*. There were only slight increases of the $InsP_5$ isomers (**22**) and (**23**) in homozygous *lpa* mutant progenies (Table 3). Altogether, cross-breeding with the commercial WT cultivar did not alter the inositol phosphate profile typical for *MIPS1* mutants.

For the *IPK1* mutant ZC-*lpa*, the impact of cross-breeding with WT cultivars on the contents of phytic acid and lower inositol phosphates was strongly influenced by the crossing parent: (i) cross-breeding of ZC-*lpa* with the WT cultivar Cu did not change the phytic acid content in the homozygous *lpa* mutant progeny (Table 4). However, due to pronounced increases of the lower inositol phosphate contents in the *lpa* mutant progenies (Table 4, Figure 2A), the total inositol phosphate contents ($InsP_2$–$InsP_6$) of the homozygous *lpa* mutant progenies were significantly higher not only than those in the *lpa* progenitor ZC-*lpa* but also than those in the crossing parent Cu and the original WT ZC-3. (ii) Upon cross-breeding of ZC-*lpa* with its original WT ZC-3, there were also significant increases of lower inositol phosphates in the homozygous *lpa* progenies. However, owing to the simultaneously reduced contents of phytic acid, the total amount of inositol phosphate was higher than in the *lpa* mutant crossing parent but lower than in the original WT ZC-3 (Table 4, Figure 2B).

In the homozygous *IPK1* mutant progeny of generation F_6 resulting from the cross-breeding with ZC-3, $Ins(1,5,6)P_3$/$Ins(3,4,5)P_3$ (**12**) and $Ins(4,5,6)P_3$ (**13**) could be quantitated for the first time and the isomers $Ins(1,4,5,6)P_4$/$Ins(3,4,5,6)P_4$ (**20**) and $Ins(1,3,4,5,6)P_5$ (**24**) were highly accumulated (Table 4). There were remarkable differences between homozygous *lpa* mutant progenies of generation F_5 and F_6 both harvested in Hangzhou, but in different growing seasons. In generation F_6 (autumn 2014), the contents of $InsP_5$ (**24**) and the $InsP_3$ isomers (**12**) and (**13**) were increased, whereas the content of $InsP_6$ was reduced by additional 44% compared to generation F_5 (spring 2014) (Table 4). These findings are in line with the different proportions of $InsP_6$ and $InsP_3$–P_5 in ZC-*lpa* mutants grown in Hangzhou in spring 2005 and autumn 2005, respectively.[14] The impact of environment (most likely temperature) may be related to the type of mutation underlying ZC-*lpa*. Sequencing has revealed that a G → A mutation affects the pre-mRNA splicing, resulting in exclusion of the fifth exon of *GmIPK1* and a disruption of the GmIKP1 functionality.[12] This pre-mRNA splicing may be influenced by the temperature in a similar way as the temperature-sensitive utilization of alternate and nonconsensus splice sites in rice genotypes with the Wx^b allele, a single-nucleotide polymorphism-controlled process eventually resulting in variation of the amylose content of rice plants grown under different temperatures.[26] It is known from field experiments that rising temperatures during maturation led to higher phytic acid accumulations in lentil and soybean

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Table 4. Inositol Phosphate Isomers (mg/g Dry Matter)[a,b] in the Crossing Parents ZC-*lpa* (*IPK1*), Cu, and ZC-3 and in Their Homozygous Wild-type (WT) and Homozygous *lpa* Mutant (*IPK1*) Progenies From Cross 1 (ZC-*lpa* × Cu), and Cross 2 (ZC-*lpa* × ZC-3), of Different Generations

	crossing parents			cross 1: ZC-*lpa* × Cu			cross 2: ZC-*lpa* × ZC-3					
	(Hangzhou, spring 2014)			(Hangzhou, spring 2014)			(Hangzhou, spring 2014)			(Hangzhou, autumn 2014)		
inositol phosphate isomer (peak no.)[c]	Cu	ZC-3	ZC-*lpa*	F_5 WT	F_5 *lpa*		F_5 WT	F_5 *lpa*		F_6 WT	F_6 *lpa*	
$InsP_2$												
$Ins(1,3)P_2$ (**1**)	–[d]	–	–	<0.13[e]	–		–	–		–	–	
$Ins(1,2)P_2$ (**2**)	–	–	–				–	–		–	–	
$Ins(4,5)P_2$ (**4**) $Ins(2,5)P_2$ (**4**)	<0.13[e]	–	–	–	–		<0.13[e]	–		<0.13[e]	–	
$InsP_3$												
$Ins(1,2,3)P_3$ (**9**) $Ins(1,2,6)P_3$ (**9**)	0.24 ± 0.01	<0.14[e]	–	<0.14[e]	–		–	–		–	–	
$Ins(1,4,6)P_3$ (**9**)												
$Ins(1,5,6)P_3$ (**12**)	–	–	<1.08[e]	–	1.40 ± 0.02		–	<1.08[e]		–	1.44 ± 0.02	
$Ins(4,5,6)P_3$ (**13**)	–	–	<1.08[e]	–	<1.08[e]		–	<1.08[e]		–	2.03 ± 0.07	
$InsP_4$												
$Ins(1,2,3,4)P_4$ (**15**) $Ins(1,3,4,6)P_4$ (**15**)	<0.05[e]	–	–	<0.05[e]	–		<0.05[e]	–		<0.05[e]	–	
$Ins(1,2,5,6)P_4$ (**18**)	<0.05[e]	<0.05[e]	–	<0.05[e]	–		<0.05[e]	–		<0.05[e]	–	
$Ins(1,4,5,6)P_4$ (**20**)	<0.05[e]	–	2.22 ± 0.12	<0.05[e]	3.05 ± 0.14			4.21 ± 0.13		<0.05[e]	4.15 ± 0.07	
$InsP_5$												
$Ins(1,2,3,4,6)P_5$ (**21**)	–	–	–	0.21 ± 0.00	–		0.14 ± 0.00	<0.73[e]		0.25 ± 0.00	–	
$Ins(1,2,3,4,5)P_5$ (**22**)	0.33 ± 0.01	0.29 ± 0.01	<0.73[e]	0.59 ± 0.00	1.07 ± 0.02		0.42 ± 0.00	<0.73[e]		0.57 ± 0.01	<0.73[e]	
$Ins(1,2,4,5,6)P_5$ (**23**)	0.32 ± 0.01	0.36 ± 0.02	<0.73[e]	0.96 ± 0.00	1.05 ± 0.04		0.98 ± 0.01	<0.73[e]		1.53 ± 0.03	<0.73[e]	
$Ins(1,3,4,5,6)P_5$ (**24**)	–	–	1.70 ± 0.04	<0.09[e]	3.11 ± 0.11		0.16 ± 0.00	2.95 ± 0.12		0.19 ± 0.00	4.74 ± 0.20	
$InsP_6$												
$Ins(1,2,3,4,5,6)P_6$ (**25**)	19.03 ± 0.14 b	19.20 ± 0.12 ab	11.50 ± 0.31 c	20.41 ± 0.11 a	11.56 ± 0.33 c		19.47 ± 0.09 a	9.85 ± 0.17 d		19.01 ± 0.07 b	5.53 ± 0.11 e	
$InsP_2$–$InsP_6$ (sum)	19.95 ± 0.15 c	19.85 ± 0.14 b	15.42 ± 0.39 d	22.19 ± 0.12 a	21.25 ± 0.44 b		21.17 ± 0.10 a	17.01 ± 0.41 d		21.57 ± 0.09 a	17.90 ± 0.09 c	

[a]Values represent means ± standard deviations resulting from duplicate analysis of two aliquots of freeze-dried flour. [b]Different letters indicate statistically significant differences (ANOVA with Tukey HSD test, $p < 0.05$) in specific inositol phosphate isomers between the two crossing parents and the corresponding progenies of different generations. [c]Only one of the possible enantiomers (Table S2) is listed. [d]Content below limit of detection (i.e., 1.1 μg of $InsP_2$, 1.2 μg of $InsP_3$, 0.5 μg of $InsP_4$, 0.8 μg of $InsP_5$, and 0.7 μg of $InsP_6$ per mL injection solution); determined on the basis of 100 μL HPIC injection volume. [e]Content below limit of quantification (mg/g dry matter), determined based on aqueous calibration solutions without consideration of the soybean matrix.

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Table 5. Inositol Phosphate Isomers (mg/g Dry Matter)[a,b] in the Crossing Parents ZC-*lpa* (*IPK1*) and TW-1-M-*lpa* (*MIPS1*) and in Their Homozygous Double Wild-type (w/w) and Homozygous Single (*MIPS1*) and Double (*IPK1*/*MIPS1*; m/m) *lpa* Mutant Progenies of Generations F_3, F_5, and F_8

	crossing parents (Hangzhou, spring 2014)		progenies F_3 (Hangzhou, spring 2014)			progenies F_5 (Hangzhou, spring 2015)		progenies F_8 (Haining, autumn 2016)	
inositol phosphate isomer (peak no.)[c]	ZC-*lpa*	TW-1-M-*lpa*	w/w	*MIPS1*	m/m	w/w	m/m	w/w	m/m
$InsP_2$									
Ins(1,3)P_2 (**1**)	–[d]	<0.13[e]	<0.13[e]	<0.13[e]	–	–	–	<0.13[e]	–
Ins(1,2)P_2 (**2**)	–	–	–	–	–	<0.13[e]	–	<0.13[e]	–
$InsP_3$									
Ins(1,2,3)P_3 (**9**) Ins(1,2,6)P_3 (**9**) Ins(1,4,6)P_3 (**9**)	–	–	–	–	–	–	–	0.23 ± 0.01	–
Ins(1,5,6)P_3 (**12**)	<1.08[e]	–	<0.14[e]	<0.14[e]	–	–	–	–	–
Ins(4,5,6)P_3 (**13**)	<1.08[e]	–	<0.14[e]	<0.14[e]	<1.08[e]	–	<1.08[e]	–	<1.08[e]
$InsP_4$									
Ins(1,2,3,4)P_4 (**15**) Ins(1,3,4,6)P_4 (**15**)	–	–	<0.05[e]	–	–	–	–	<0.05[e]	–
Ins(1,2,5,6)P_4 (**18**)	–	–	<0.05[e]	–	–	<0.05[e]	–	<0.05[e]	–
Ins(1,4,5,6)P_4 (**20**)	2.22 ± 0.12	–	0.31 ± 0.00	0.05 ± 0.00	0.32 ± 0.01	<0.05[e]	0.44 ± 0.04	<0.05[e]	0.54 ± 0.02
$InsP_5$									
Ins(1,2,3,4,6)P_5 (**21**)	–	<0.09[e]	0.15 ± 0.00	<0.09[e]	–	<0.09[e]	–	–	–
Ins(1,2,3,4,5)P_5 (**22**)	<0.73[e]	0.15 ± 0.00	0.38 ± 0.01	0.20 ± 0.01	–	0.32 ± 0.01	–	0.23 ± 0.00	–
Ins(1,2,4,5,6)P_5 (**23**)	<0.73[e]	0.20 ± 0.00	0.74 ± 0.01	0.29 ± 0.01	–	0.40 ± 0.01	–	0.33 ± 0.01	–
Ins(1,3,4,5,6)P_5 (**24**)	1.70 ± 0.04	<0.09[e]	0.24 ± 0.00	0.18 ± 0.00	1.30 ± 0.04	<0.09[e]	1.17 ± 0.02	–	1.99 ± 0.07
$InsP_6$									
Ins(1,2,3,4,5,6)P_6 (**25**)	11.50 ± 0.31 c	9.90 ± 0.06 d	16.93 ± 0.04 b	7.40 ± 0.07 e	3.87 ± 0.08 g	18.31 ± 0.08 a	4.63 ± 0.07 f	18.20 ± 0.07 a	2.32 ± 0.03 h
$InsP_2$–$InsP_6$ (sum)	15.42 ± 0.39 b	10.25 ± 0.05 c	18.74 ± 0.06 a	8.12 ± 0.07 d	5.49 ± 0.08 f	19.02 ± 0.08 a	6.24 ± 0.06 e	19.01 ± 0.08 a	4.85 ± 0.05 g

[a]Values represent means ± standard deviations resulting from duplicate analysis of two aliquots of freeze-dried flour. [b]Different letters indicate statistically significant differences (ANOVA with Tukey HSD test, $p < 0.05$) in specific inositol phosphate isomers between the two crossing parents and the corresponding progenies. [c]Only one of the possible enantiomers (Table S2) is listed. [d]Content below limit of detection (i.e., 1.1 μg of $InsP_2$, 1.2 μg of $InsP_3$, 0.5 μg of $InsP_4$, 0.8 μg of $InsP_5$, and 0.7 μg of $InsP_6$ per mL injection solution); determined on the basis of 100 μL HPIC injection volume. [e]Content below limit of quantification (mg/g dry matter), determined based on aqueous calibration solutions without consideration of the soybean matrix.

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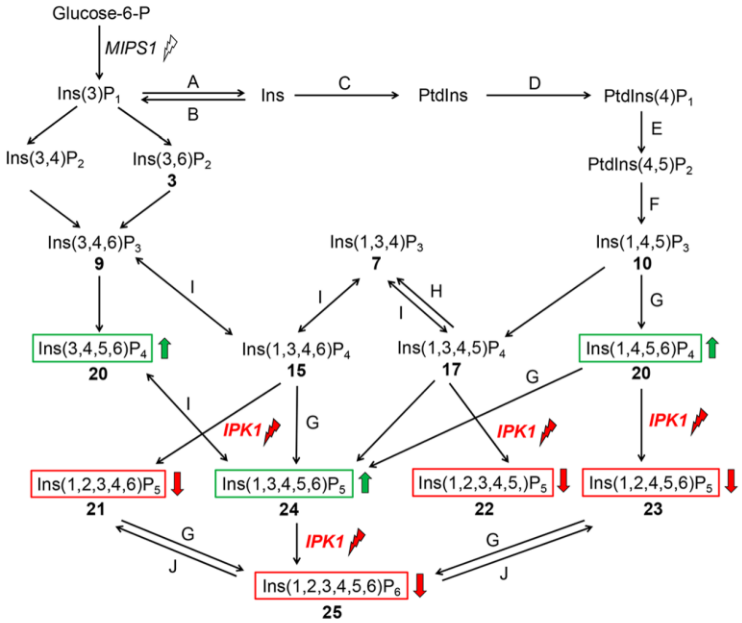

Figure 1. Simplified biosynthetic pathway of phytic acid in eukaryotic cells[1] and biochemical consequences of the *MIPS1* (D-*myo*-inositol 3-phosphate synthase 1) and *IPK1* (inositol 1,3,4,5,6 pentakisphosphate ($InsP_5$) 2-kinase) mutations. The left side shows the lipid-independent pathway and the right side the lipid-dependent pathway. Enzymes catalyzing critical steps are illustrated: A, Phosphatase; B, Ins Kinase; C, Phosphatidylinositol (PtdIns) Synthase; D, PtdIns 4-Kinase; E, PtdIns(4)P_1 5-Kinase; F, Phospholipase C; G, $InsP_3$ 3-/5-/6-Kinase; H, 5-Phosphatase; I, Ins PolyP Kinase/Phosphatase; J, Phytase. Inositol phosphate isomers determined with the applied HPIC-method are numbered according to Table S2.

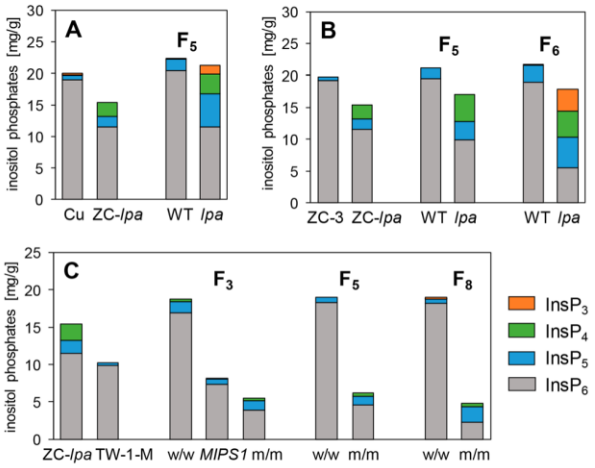

Figure 2. Mean contents (mg/g dry matter) and distributions of phytic acid ($InsP_6$) and lower inositol phosphates ($InsP_3$–$InsP_5$) in the crossing parents and in the corresponding homozygous wild-type (WT) and homozygous *lpa* mutant (*lpa*) progenies of different generations from the crosses (A) ZC-*lpa* × ZC-3, (B) ZC-*lpa* × Cu, and (C) ZC-*lpa* × TW-1-M-*lpa* (w/w, homozygous double wild-type progeny; m/m, homozygous double *lpa* mutant (*IPK1*/*MIPS1*) progeny; *MIPS1*, homozygous *MIPS1* mutant progeny).

seeds.[27] Altogether, the data suggest that rising temperatures during spring time and declining temperatures during autumn, resulting in different temperature profiles during seed development, might at least partly explain the differences in $InsP_6$ and lower inositol phosphate contents between *IPK1*-progenies of generation F_5 and F_6. These phenomena were also seen for the wild-type progenies but to a lower extent than in the *lpa* mutant progenies (Figure 2B).

Generation of a Double *lpa* Mutant by Crossing of ZC-*lpa* (*IPK1*) with TW-1-M-*lpa* (*MIPS1*). The reductions in phytic acid contents observed in the double *lpa* mutants were significantly more pronounced than those expected from the single mutants. In addition, only isomers that were present at high concentrations in the single *IPK1* mutant ZC-*lpa*, like $InsP_4$ (**20**) and $InsP_5$ (**24**), could be quantitated in the double mutants. The *IPK1* mutation led to an accumulation of the direct precursors of phytic acid in homozygous double *lpa* mutant progenies but to a lower extent than the single *IPK1* mutant due to the additional interruption in the supply of Ins(3)P_1 by the *MIPS1* mutation (Figure 1). Therefore, the homozygous double *lpa* mutant progenies showed both the lowest phytic acid contents and the lowest amounts of total inositol phosphates of all soybean samples investigated in the present study (Table 5, Figure 2C). The described phytic acid contents in double mutants simultaneously carrying the *MIPS1* and the *IPK1* mutation are comparable with those of soybean double mutants with two *IPK1* mutations (14IPK1 and 06IPK1)[16] or with those described for *Arabidopsis* double *lpa* mutant seeds with disrupted inositol polyphosphate kinases *AtIPK1* and *AtIPK2β*.[28]

Owing to limitations in sample amounts, inorganic phosphorus (Pi) was not investigated in the course of the present study. It is known from literature that the total P content both in *MIPS1* and *IPK1* mutant lines remains similar to their respective WT parents.[13] Therefore, it can be assumed that all investigated *lpa* samples accumulated Pi to keep the total P content on a level comparable to their respective WTs.

Biosynthetic Considerations. Three principle pathways have been proposed for the biosynthesis of phytic acid in

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eukaryotic cells.[28] The lipid-dependent pathways result from the hydrolysis of PtdIns(4,5)P_2 by Phospholipase C and subsequent phosphorylations via Ins(1,4,5,6)P_4 (**20**) (pathway I), Ins(1,3,4,5)P_4 (**17**), or Ins(1,3,4,6)P_4 (**15**) (pathway II) (Figure 1, right side). The lipid-independent pathway III involves the consecutive phosphorylation of Ins(3)P_1 or *myo*-inositol (Figure 1, left side).[28]

The enzyme *IPK1* (inositol 1,3,4,5,6 penta*kis*phosphate 2-kinase) catalyzes the last step in the biosynthesis of phytic acid. However, results from in vitro characterizations of recombinant *IPK1* enzymes and analyses of different *IPK1* mutants indicate that plant *IPK1* genes encode a polyphosphate kinase that can use different $InsP_3$ and $InsP_4$ isomers besides Ins(1,3,4,5,6)P_5 (**24**) as substrates, including Ins(1,4,6)P_3 (**9**) and Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 (**20**).[16,29−31] Therefore, the mutation of the *IPK1* gene not only affects the formation of phytic acid but also leads to an accumulation of the inositol phosphate isomers Ins(1,5,6)P_3/Ins(3,4,5)P_3 (**12**), Ins(4,5,6)-P_3 (**13**), Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 (**20**), and Ins-(1,3,4,5,6)P_5 (**24**), which cannot be utilized to produce higher inositol phosphates (Figure 1).

In an *IPK1* mutant of *Arabidopsis thaliana*, Ins(1,3,4,5,6)P_5 (**24**) and Ins(1,4,5,6)P_4 and/or Ins(3,4,5,6)P_4 (**20**) and other nonidentified $InsP_3$ isomers (not Ins(1,4,5)P_3) were accumulated compared to its respective wild-type.[32] Sun and Jaisi (2018) reported Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 (**20**) to be a minor $InsP_4$ isomer in commercial soybean cultivars besides the dominating isomers Ins(1,2,4,5)P_4/Ins(2,3,5,6)P_4 (**16**) and Ins(1,2,5,6)P_4/Ins(2,3,4,5)P_4 (**18**).[17] However, in the present study the $InsP_4$ isomer (**20**) was a major representative in *lpa* soybean mutants carrying the *IPK1* mutation target. An accumulation of $InsP_4$ (**20**) has also been reported in *lpa*2−2 maize seeds besides increased levels of several $InsP_5$ and $InsP_3$ isomers.[33]

The $InsP_3$ isomers (**12**) and (**13**) were reported in the *IPK1* mutant ZC-*lpa* and in their corresponding homozygous *lpa* mutant progenies after cross-breeding with WT cultivars for the first time (Tables 2 and 4). Trace amounts of (**12**)[17] and (**13**)[18] have already been reported in commercial soybean samples. So far, biochemical routes leading to their formation have not been described. Their synthesis cannot be explained by hydrolysis of any of the known members of the phosphoinositide family found in eukaryotic cells, i.e., PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,5)P_2, PtdIns-(3,4)P_2, and PtdIns(4,5)P_2, PtdIns(3,4,5)P_3.[34] Therefore, it is unlikely that their formation is lipid-dependent. For Ins-(1,5,6)P_3 (**12**), which is the enantiomer of Ins(3,4,5)P_3 (**12**), the following hypothetical sequence could be possible: (i) enzymatic hydrolysis of PtdIns(5)P resulting in Ins(1,5)P_2 and (ii) subsequent phosphorylation at position 6. Notwithstanding the present lack of knowledge regarding their potential biogenesis, a consecutive phosphorylation of the described $InsP_3$ isomers could result in the higher inositol phosphates found in soybean seeds carrying the *IPK1* mutation target, e.g., the phosphorylation of Ins(1,5,6)P_3 (**12**) might lead to Ins(1,4,5,6)P_4 (**20**), Ins(1,2,4,5,6)P_5 (**23**), and finally Ins-(1,2,3,4,5,6)P_6 (**25**).

In most eukaryotic cells including plant vegetative tissues, the lipid-dependent pathway represents the major route to the formation of phytic acid (Figure 1, right side). For seeds, however, the lipid-independent pathway seems to be more important.[35] The results of this study support the conclusion that both the lipid-dependent (through Ins(1,4,5,6)P_4) and the lipid-independent routes (through Ins(3,4,5,6)P_4) are involved in the phytic acid biosynthesis in soybean seeds[16] (Figure 1). The detection of inositol phosphate isomers hitherto not reported in *lpa* soybeans might help to further elucidate the biosynthetic pathways leading to phytic acid.

Physiological and Nutritional Aspects. Several physiological functions have been described for inositol phosphates in eukaryotes.[36] For example, Ins(1,4,5)P_3 (**10**) has been identified as a second messenger that mediates receptor-induced Ca^{2+} mobilization,[37] but in higher plants this isomer was shown to only be a minor $InsP_3$.[38] Ins(1,3,4,5)P_4 (**17**) has been found to play important roles in T cell, B cell, and neutrophil cell development and function[39] and in natural killer (NK) cell maturation and responsiveness.[40] Ins(3,4,5,6)-P_4 (**20**) inhibits Cl^- conductance through the chloride channel ClC-3 in vivo and thus has been discussed to possess the potential to regulate neuronal development, tumor cell migration, bone remodeling, apoptosis, and inflammatory responses.[41] For Ins(1,3,4,5,6)P_5 (**24**), a range of physiological properties including antiangiogenic, pro-apoptotic, and anti-cancer activities have been described in vitro and in vivo.[42,43]

The contribution of dietary inositol phosphates to the levels found in the human body is controversially discussed, and no carrier has been identified in the gut so far.[36] However, results from studies in animals and humans indicate that intracellular inositol phosphate levels in various tissues and biological fluids as well as urinary excretion are related to oral phytic acid intake.[44,45] Notwithstanding knowledge gaps regarding bioavailability and dose−response relationships of lower inositol phosphates in animals and humans, the *IPK1* soybean mutants with their high contents of $InsP_3$−$InsP_5$ might be a valuable source of these bioactive compounds.

The mineral binding capacity of inositol phosphates is lowered with decreasing number of phosphate groups per molecule, and the formed complexes of such lower inositol phosphates exhibit higher solubility.[46] In suckling rats, the zinc and calcium uptake was inhibited by $InsP_6$ and $InsP_5$ but not by $InsP_4$ and $InsP_3$.[47] In humans, $InsP_3$ and $InsP_4$ also showed no inhibitory effect on iron absorption, if tested separately.[48] However, in mixtures containing inositol phosphates with differing phosphorylation stages, lower inositol phosphates can contribute to the inhibitory effect of mineral absorption, possibly by forming mineral complexes between different inositol phosphate isomers.[48] Therefore, the accumulations of $InsP_3$−$InsP_5$ in *IPK1* mutant soybean seeds might to a minor degree antagonize the positive effect of the phytic acid reduction on mineral bioavailability in humans and livestock.

Agronomic Performance. Double *lpa* mutant soybean lines carrying two independent *IPK1* mutations (14IPK1 and 06IPK1) did not show significant reductions in germination or field emergence compared to their respective wild-types.[16] Due to the limited amount of sample material, the agronomic performance of progenies resulting from the cross-breeding experiments performed in the present investigation could not be evaluated. A previous assessment has not revealed a significant influence of the *IPK1* mutation on field emergence or yield of ZC-*lpa* and its progenies in a wide range of growing environments.[13] Therefore, it might be assumed that the ZC-*lpa* F_6 progenies and the F_8 progenies of the double mutant, showing exceptionally low $InsP_6$ contents of 5.5 and 2.3 mg/g, respectively (Tables 4 and 5), would also not show major agronomic disadvantages. Larger field trials yielding more plant

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materials would be necessary to draw final conclusions regarding their agronomic competitiveness.

In summary, the data demonstrate that the phytic acid and the lower inositol phosphate contents in homozygous *lpa* mutant progenies are influenced by the type of mutation, the crossing parent, and the environmental conditions. The typical inositol phosphate signature of the *IPK1* soybean mutant, i.e., reduced $InsP_6$ content and accumulation of lower inositol phosphates, was not altered by crossing with commercial cultivars. *IPK1* progenies showed pronounced additional accumulations of specific $InsP_3$–$InsP_5$ isomers compared with the progenitor *lpa* mutant. The choice of the WT crossing parent had a significant influence on the extent of $InsP_6$ reduction and thus on the total amount of inositol phosphates. Crossing with the original WT was shown to be more powerful to generate homozygous *lpa* mutant progenies with particularly low $InsP_6$ contents.

The generation of double *lpa* mutants simultaneously carrying *MIPS1* and *IPK1* mutation targets offers an option to drastically reduce the $InsP_6$ content accompanied by only moderate increases in lower inositol phosphates. Notwithstanding knowledge gaps regarding the underlying mechanisms, the elaborated data show that the application of cross-breeding approaches offers the potential to modulate the amounts of both $InsP_6$ and lower inositol phosphates in *lpa* mutants and thus to influence their nutritional quality.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b06117.

Soybean sample material; overview of inositol phosphate isomers separated by HPIC; structure of *myo*-inositol and overview of all possible *myo*-inositol phosphates; LODs, LOQs, and characteristics of calibration curves; method validation; sequence of *GmIPK1* and *GmMIPS1* genes; HPIC chromatograms of the reference standard solution and ZC-*lpa*; genotyping results by CADMA-HRM analysis (PDF)

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Notes

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■ ABBREVIATIONS USED

CADMA, competitive amplification of differentially melting amplicons analysis; ClC-3, chloride channel 3; HPIC, high pressure ion chromatography; HRM, high-resolution melting; $InsP_2$–$InsP_5$, lower inositol phosphates; $InsP_6$, phytic acid; *IPK1*, inositol 1,3,4,5,6-penta*kis*phosphate 2-kinase; *lpa*, low phytic acid; *MIPS1*, D-*myo*-inositol 3-phosphate synthase gene 1; PtdIns, phosphatidylinositol; WT, wild-type

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3.2 Publication II

Goßner, S.; Yuan, F.; Zhou, C.; Tan, Y.; Shu, Q.; Engel, K. H.
Stability of the Metabolite Signature Resulting from the *MIPS1* Mutation in *Low Phytic Acid* Soybean (*Glycine max* L. Merr.) Mutants upon Cross-Breeding.
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In the *lpa* soybean mutant *Gm-lpa*-TW-1-M, *γ*-irradiation had resulted in a 2 bp deletion in the third exon of the *myo*-inositol 3-phosphate synthase (*MIPS1*) gene. This *lpa* mutant was crossed with a commercial WT cultivar to investigate the potential impact of such cross-breeding steps on the metabolite profiles of the homozygous WT and homozygous *lpa* mutant progenies. The individual F_3 and F_5 progenies as well as the crossing parents were subjected to a non-targeted GC-based metabolite profiling approach allowing the analysis of a broad spectrum of polar and lipophilic low molecular weight constituents. In homozygous *lpa* mutant progenies, the *MIPS1* mutation resulted not only in the intended reduction of phytic acid, but also in remarkable changes of other nutritionally relevant constituents, i.e. reduced concentrations of *myo*-inositol, raffinose, stachyose, galactopinitol A, galactopinitol B, and ciceritol as well as increased concentrations of sucrose and various free amino acids compared to the respective wild-type progenies. The mutation-induced metabolite signature was neither compromised by the cross-breeding step nor by fluctuations of metabolite levels in progenies observed in different generations and growing seasons. The elaborated data demonstrate that the *MIPS1 lpa* soybean mutant *Gm-lpa*-TW-1-M as well as its homozygous *lpa* mutant progenies might be valuable genetic resources for the commercial production of high quality soybean seeds consistently possessing desirable phytate- and carbohydrate digestability-related traits.

Candidate's contribution:
Performance of the complete sample work-up sequence required for the analysis of all soybean samples; performance of the GC/MS and GC/FID-based metabolite profiling for all investigated soybean samples; implementation and evaluation of quality control

analyses for the metabolite profiling approach; analysis of phytic acid in all soybean seeds by high pressure ion chromatography (HPIC); data processing and multivariate and univariate statistical assessment of the elaborated metabolite profiling and HPIC data and interpretation of the results; writing and revision of the complete manuscript including all Figures and Tables as well as the Supporting Information.

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Stability of the Metabolite Signature Resulting from the *MIPS1* Mutation in *Low Phytic Acid* Soybean (*Glycine max* L. Merr.) Mutants upon Cross-Breeding

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Supporting Information

ABSTRACT: The *low phytic acid* (*lpa*) soybean (*Glycine max* L. Merr.) mutant *Gm-lpa*-TW-1-M, resulting from a 2 bp deletion in *GmMIPS1*, was crossed with a commercial cultivar. F_3 and F_5 progenies were subjected to nontargeted GC-based metabolite profiling, allowing analysis of a broad array of low molecular weight constituents. In the homozygous *lpa* mutant progenies the intended phytic acid reduction was accompanied by remarkable metabolic changes of nutritionally relevant constituents such as reduced contents of raffinose oligosaccharides and galactosyl cyclitols as well as increased concentrations in sucrose and various free amino acids. The mutation-induced metabolite signature was nearly unaffected by the cross-breeding and consistently expressed over generations and in different growing seasons. Therefore, not only the primary *MIPS1 lpa* mutant but also its progenies might be valuable genetic resources for commercial breeding programs to produce soybean seeds stably exhibiting improved phytate-related and nutritional properties.

KEYWORDS: *metabolite profiling, low phytic acid (lpa) mutant, soybean (Glycine max L. Merr.), cross-breeding, MIPS1*

INTRODUCTION

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, $InsP_6$) and its mixed cationic salts (phytates) are the major storage form of inorganic phosphorus (P) in mature seeds like grains, legumes, oilseeds, and nuts, constituting 65−85% of total P.[1] For soybeans (*Glycine max* L. Merr.), $InsP_6$ contents between 1.0% and 4.6% have been reported.[2,3] Depending on the proportion of foods with high phytate contents in the diet, the dietary intake of phytate for adults may range between about 180 and 4500 mg/d.[4] Under physiological conditions, phytic acid is strongly negatively charged; therefore, it can bind proteins and form indigestible chelates with di- and trivalent cations like calcium, magnesium, zinc, and iron. This may reduce their digestibility and bioavailability[5] and, in the case of imbalanced diets with cereals and legumes as staple foods, lead to serious deficiencies.[6] In addition, the excretion of undigested $InsP_6$ leads to phosphorus pollution and eutrophication of waterways.[7] One approach to meet these problems is the generation of *low phytic acid* (*lpa*) crops.[8]

For soybeans, *lpa* crops have been obtained through chemical[9,10] or physical mutagenesis[11] and by genetic engineering via transformation or gene silencing.[12−14] The *lpa* mutant LR33 had been generated by chemical mutagenesis, resulting in a single base change in the *myo*-inositol 3-phosphate synthase (*MIPS1*) gene. In addition to the intended effect of phytic acid reduction, targeted analysis showed lower levels of *myo*-inositol, raffinose, and stachyose as well as an increased sucrose content compared to its original wild-type.[10] *Gm-lpa*-TW75−1 (TW-*lpa*) and its natural mutant *Gm-lpa*-TW-1-M (TW-1-M-*lpa*) have been generated via γ-irradiation of Taiwan 75, resulting in a 2 bp deletion in the third exon of *MIPS1*.[11,15] For TW-*lpa*, targeted analysis revealed lowered stachyose and raffinose as well as increased sucrose contents.[16] The correlation between the *MIPS1* mutation and the resulting metabolic phenotype has also been demonstrated through the application of an untargeted GC-based metabolite profiling approach.[17] The intended reduction of phytic acid of 53% owing to the *MIPS1* mutation in the *lpa* mutant TW-*lpa* was shown to be accompanied by several consistent metabolic changes, e.g., reduced contents of raffinose oligosaccharides (RFOs) and galactosyl cyclitols as well as increased concentrations of various amino acids.

Lpa mutant lines often show inferior agronomic performance, and cross-breeding is being used to generate *lpa* cultivars with superior agronomic traits.[18] However, except for one recent study on the stability of the metabolite signature resulting from the *OsSULTR3;3* mutation in *lpa* rice seeds upon cross-

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breeding,[19] data regarding the potential impact of such crossing steps on the metabolite profiles of the *lpa* mutant progenies are missing.

Therefore, homozygous wild-type and *lpa* mutant F_3 and F_5 populations were generated by crossing the *MIPS1 lpa* mutant TW-1-M-*lpa* with the commercial cultivar Zhexiandou No. 4 (ZXD). The objectives of the study were (i) to investigate the impact of cross-breeding with a commercial wild-type on the *MIPS1* mutation-induced metabolite signature of the homozygous *lpa* mutant progenies and (ii) to assess the stability of their metabolic phenotype depending on generations and growing seasons.

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 obtained from Fluka (Buchs, Switzerland). Reference compounds were supplied by VWR International (Darmstadt, Germany), Fluka (Buchs, Switzerland), Sigma-Aldrich (Steinheim, Germany), and Roth (Karlsruhe, Germany). All other reagents and solvents were of analytical grade.

Sample Materials. The *lpa* soybean mutant *Gm-lpa*-TW75−1 (TW-*lpa*) had been generated by treating the wild-type Taiwan 75 with γ-irradiation (150 Gy).[11] In this study, *Gm-lpa*-TW-1-M (TW-1-M-*lpa*), a natural mutant of TW-*lpa* also possessing a 2 bp deletion in *MIPS1* but exhibiting higher germination percentage and speed, better storage stability, and a higher rate of field emergence,[15] was used. This *lpa* mutant was crossed with Zhexiandou No. 4 (ZXD), a wild-type cultivar widely adapted to various Chinese provinces.[20] A flowchart outlining the procedure applied to generate homozygous wild-type and *lpa* mutant F_3 and F_5 soybean seeds is presented in Figure 1. F_1 plants were grown in spring (from April to July) 2013. F_2 plants (as well as the original crossing parents) were grown in spring 2014, genotyped using allele-specific molecular markers,[11,21] and classified into homozygous wild-type (HWT), homozygous *lpa* mutant (HM), and heterozygous ones. The F_3 seeds from heterozygous F_2 plants were planted in autumn (from August to October) 2014 and again genotyped for identification of heterozygous F_3 plants. The F_4 seeds of individual heterozygous F_3 plants were grown into F_4 populations for identification of homozygous wild-type and *lpa* mutant plants in autumn 2015. By starting from two independent heterozygous F_3 plants, two individual F_5 lines of wild-type and *lpa* mutant, respectively, were obtained. All field trials were performed in Hangzhou, China; the same standard agronomic practices were applied.

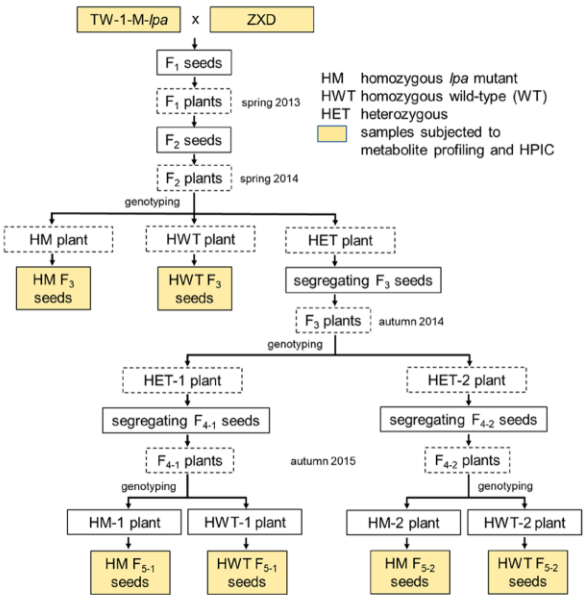

Figure 1. Flowchart of cross-breeding steps to produce the investigated soybean materials. All samples were planted in Hangzhou, China.

The soybean seeds were freeze-dried for 24 h and subsequently ground with a cyclone mill equipped with a 500 μm sieve. The flour was again freeze-dried (48 h) and stored in LDPE bottles (−18 °C).

Genotyping of Progenies. For every generation, segregating plants were genotyped using DNA extracts of leaf tissues.[21] The extracts were used for asymmetric PCRs with competitive primers to discriminate homozygous *lpa MIPS1* mutants, homozygous WTs, and heterozygous progenies by high-resolution melting (HRM) curves (Figure S1).

Analysis of Phytic Acid. The phytic acid contents of the soybean materials were determined by high-pressure ion chromatography (HPIC) according to a formerly reported protocol.[22]

Metabolite Profiling. *Preparation of Retention Time Standards and Internal Standards.* Retention time standards were prepared by dissolving the hydrocarbons undecane, hexadecane, tetracosane, triacontane, and octatriacontane in *n*-hexane at the previously described concentrations.[17] The following internal standards were used: 1.5 mg/mL tetracosane in *n*-hexane (fraction I), 0.3 mg/mL 5α-cholestan-3β-ol in dichloromethane (fraction II), 0.8 mg/mL phenyl-β-D-glucopyranoside in deionized water (fraction III), and 0.3 mg/mL *p*-chloro-L-phenylalanine in deionized water (fraction IV).

Sample Workup. Metabolite profiling of freeze-dried soybean flour was performed according to a previously reported extraction and fractionation scheme.[17] Three aliquots (300 mg) of flour were weighted into disposable cartridges and soaked (20 min) in 200 mL of methanol for cell digestion. Methanol was removed under vacuum (25 mbar, 30 min), and nonpolar and polar metabolites were extracted consecutively 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 internal standard solutions I and II as well as 250 μL of internal standard solutions III and IV were added to the nonpolar and polar extract, respectively. The dried nonpolar extract was transesterified with sodium methoxide, and after evaporation to dryness the redissolved lipids were separated by solid-phase extraction (SPE) into fractions I and II. Fraction II was silylated with 50 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (70 °C, 15 min) before GC analysis. One milliliter of the filtered polar extract was evaporated to dryness and silylated with 100 μL of trimethylsilylimidazole (70 °C, 20 min). Fraction III (sugars and sugar alcohols) was obtained by selective hydrolysis of the silylated derivatives. Furthermore, 2 mL of the dried polar extract was subjected to oximation (300 μL of hydroxylamine hydrochloride, 70 °C, 30 min), silylation (50 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide, 70 °C, 20 min), and selective hydrolysis to separate a fraction containing organic and inorganic acids, amino acids, and amines. One microliter of each fraction was analyzed by capillary gas chromatography (GC) coupled with a flame ionization detector (FID) and a mass spectrometer (MS).[23] In addition, a 1:10 dilution of fraction I was injected to quantitate highly concentrated constituents.

Metabolites were identified by comparing chromatographic and mass spectral data with those of reference compounds, with data from the NIST08 mass spectra library and with the literature.[17]

Quality Control. Commercially available soybeans were used as reference material. They were subjected to the analytical procedure in regular intervals together with actual samples to ensure constant analytical conditions and to control potential laboratory contamination. For each fraction, representative metabolites were used to evaluate the acceptability of the results. Data were accepted if the relative standard deviations of the selected compounds did not exceed a threshold of 20%.

Data Processing and Statistical Analysis. Each biological replicate was analyzed in triplicate. The GC/FID data were acquired and integrated using Chrom-Card 2.11 (Thermo Fisher Scientific,

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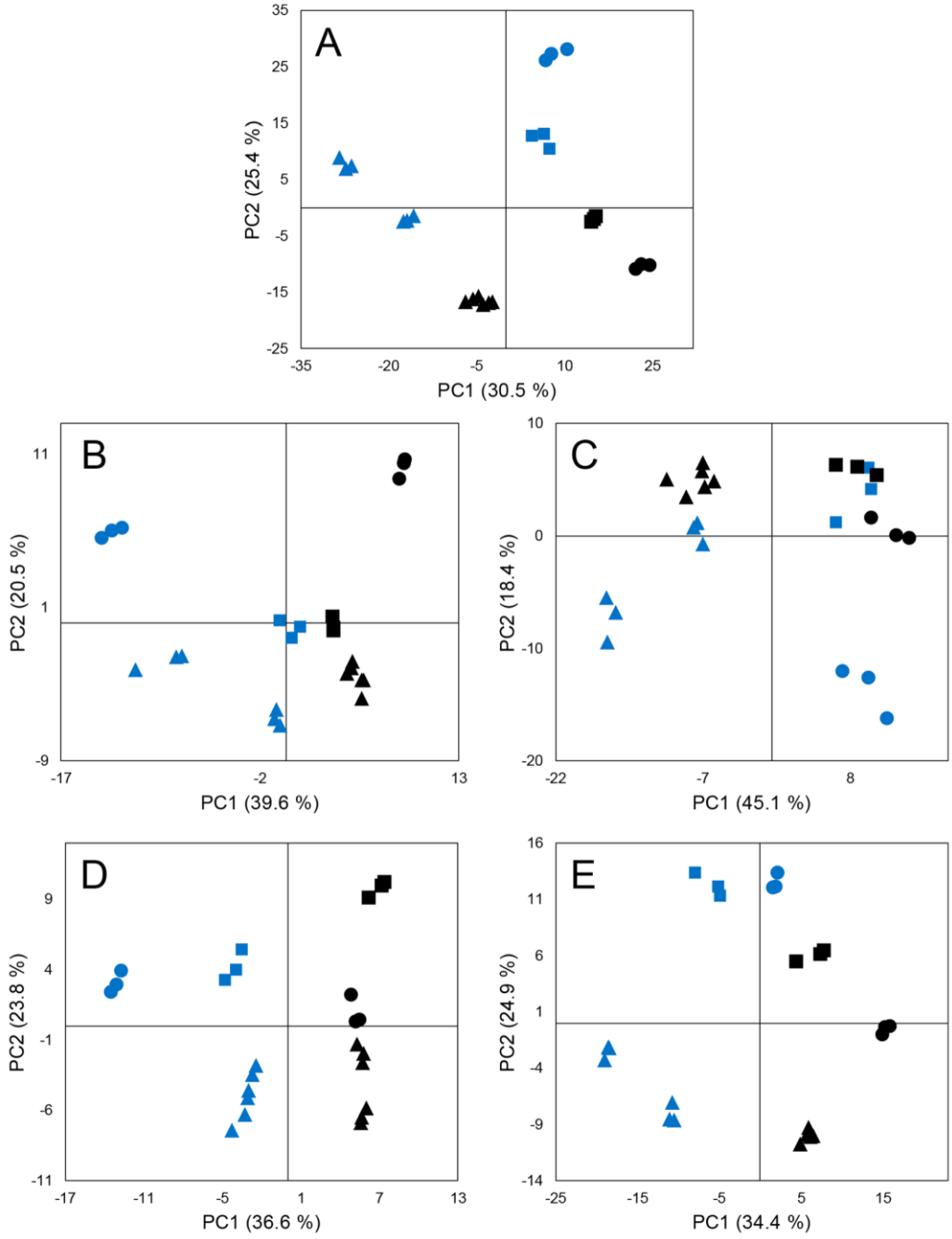

Figure 2. PCA score plots of metabolite profiling data of combined fractions I–IV (A) and single fractions I (B), II (C), III (D), and IV (E) from homozygous wild-type (black) and homozygous *lpa* mutant (blue) soybean seeds: TW-1-M-*lpa* and WT crossing parent ZXD (circles), homozygous F_3 (squares), and F_5 (triangles) progenies.

Waltham, MA, USA). Retention times were aligned using the retention time standards, and the peak heights were standardized based on the internal standards by Chrom*pare* 1.1 (http://www.chrompare.com). Peaks below noise level were discarded on the basis of a threshold of 1% peak height relative to the internal standards. GC/MS data acquisition and integration was performed using Xcalibur 2.1 (Thermo Fisher Scientific, Waltham, MA, USA).

For metabolite profiling data pretreatment, cube root transformation and Pareto scaling were conducted using the web-based tool MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml).[24] Principal component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were performed using MetaboAnalyst 4.0, and the OPLS-DA model was validated by a 10-fold cross validation and permutation tests (n = 2000).[24] Variable importance in projection (VIP) values of the OPLS-DA model were calculated using Workflow4metabolomics 3.2 (https://galaxy.workflow4metabolomics.org).

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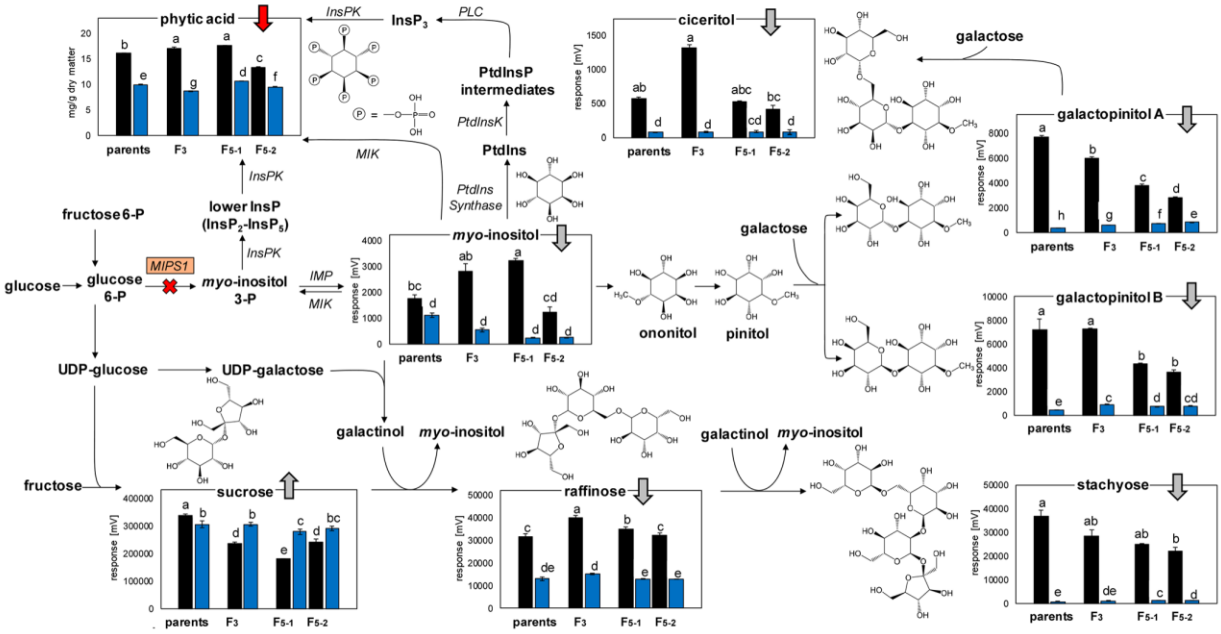

Figure 3. Simplified biosynthetic pathway indicating changes in the metabolite profiles observed for the crossing parents (ZXD vs TW-1-M-*lpa*) and their F_3 and F_5 progenies (homozygous wild-type vs homozygous *lpa*). For each metabolite, different letters indicate statistically significant differences in relative peak intensities (ANOVA with multiple pairwise comparisons by Tukey's post-hoc test, Kruskal–Wallis with Conover–Iman post-hoc test and Bonferroni correction, or Welch-ANOVA with Games-Howell post-hoc test; $p < 0.05$, respectively). *MIPS1*, 1D-*myo*-inositol 3-phosphate synthase; *IMP*, *myo*-inositol monophosphatase; *MIK*, *myo*-inositol kinase; *PtdIns Synthase*, phosphatidylinositol synthase; *PtdInsK*, phosphatidylinositol phosphate kinase; *PLC*, phospholipase C; *InsPK*. inositol phosphate kinase.

For univariate comparisons, XLSTAT version 2018.7 (Addinsoft, Paris, France) was utilized. If data were normally distributed and homoscedastic, they were analyzed by Student's *t*-test and analysis of variance (ANOVA), followed by pairwise comparison by Tukey's post-hoc test. Heteroscedastic, normally distributed data were compared with Welch's *t*-test and Welch-ANOVA, followed by Games–Howell post-hoc test. Mann–Whitney test and Kruskal–Wallis test with Conover–Iman post-hoc test and Bonferroni correction were performed for not normally distributed data. The significance level was set to $p < 0.05$ for all statistical comparisons.

RESULTS

A *low phytic acid* soybean mutant (TW-1-M-*lpa*) was crossed with a commercial cultivar (ZXD), and the progenies, all grown in the same experimental field, were classified based on allele-specific molecular markers[21] into homozygous wild-type, homozygous *lpa* mutant, and heterozygous plants. According to the genotyping results, two individual heterozygous F_3 plants were selected for production of segregating F_4 plants. Genotyping was again used to select individual homozygous wild-type and *lpa* mutant plants and to obtain the corresponding homozygous F_5 seeds (Figure 1).

The applied metabolite profiling procedure resulted in four fractions containing FAMEs and hydrocarbons (fraction I), FFAs, fatty alcohols, tocopherols, triterpenic alcohols, and sterols (fraction II), sugars and sugar alcohols (fraction III), and amino acids, amines, and organic and inorganic acids (fraction IV). A total of 499 peaks was detected, of which 81 were identified in the lipophilic and 77 in the polar fractions. The respective chromatographic and mass spectral data are provided in the Supporting Information (Tables S1–S4).

Multivariate Analysis of Soybean Metabolite Profiles. In the PCA score plot of the combined fractions I–IV, a clear separation along PC2 was observable for the parents TW-1-M-*lpa* and ZXD as well as for the homozygous wild-type and *lpa* mutant F_3 and F_5 progenies (Figure 2A). The separations of the progenies were less pronounced than those of the crossing parents. There was an additional pronounced shift along PC1 and PC2 between F_3 and F_5 progenies which had been grown in different seasons. However, this shift did not compromise the clear separations between homozygous WT and *lpa* mutant progenies.

PCA score plots of the single fractions are presented in Figure 2B–E. In accordance with previous findings in Taiwan 75 and its *MIPS1 lpa* soybean mutant TW-*lpa*,[17] the separation of wild-types and *lpa* mutants was mainly driven by polar compounds of fractions III and IV (Figure 2D and 2E). In fraction II (Figure 2C), only the parents could be clearly separated whereas the homozygous wild-type and *lpa* F_3 and F_5 progenies, respectively, clustered together. Except for fraction I, distinct separations of F_3 and F_5 lines were observed in all fractions.

For the seeds of the two individual plants available for F_5, the homozygous *lpa* progenies were clearly separated, whereas the corresponding homozygous WT progenies clustered together (Figure 2A, 2B, 2C, and 2E).

Metabolic Differences between Homozygous Wild-Type and Homozygous *lpa* Mutant Progenies. In order to identify constituents responsible for the discrimination seen in the combined PCA score plot (Figure 2A), the corresponding loading plot (Figure S2) and peak-by-peak comparisons were employed.[25] The loading plot indicated differences in the concentrations between wild-type and *lpa* progenies for various metabolites, including members of the raffinose family oligosaccharides (RFOs) and galactosyl cyclitols. These metabolites are closely linked to the biosynthesis of phytic

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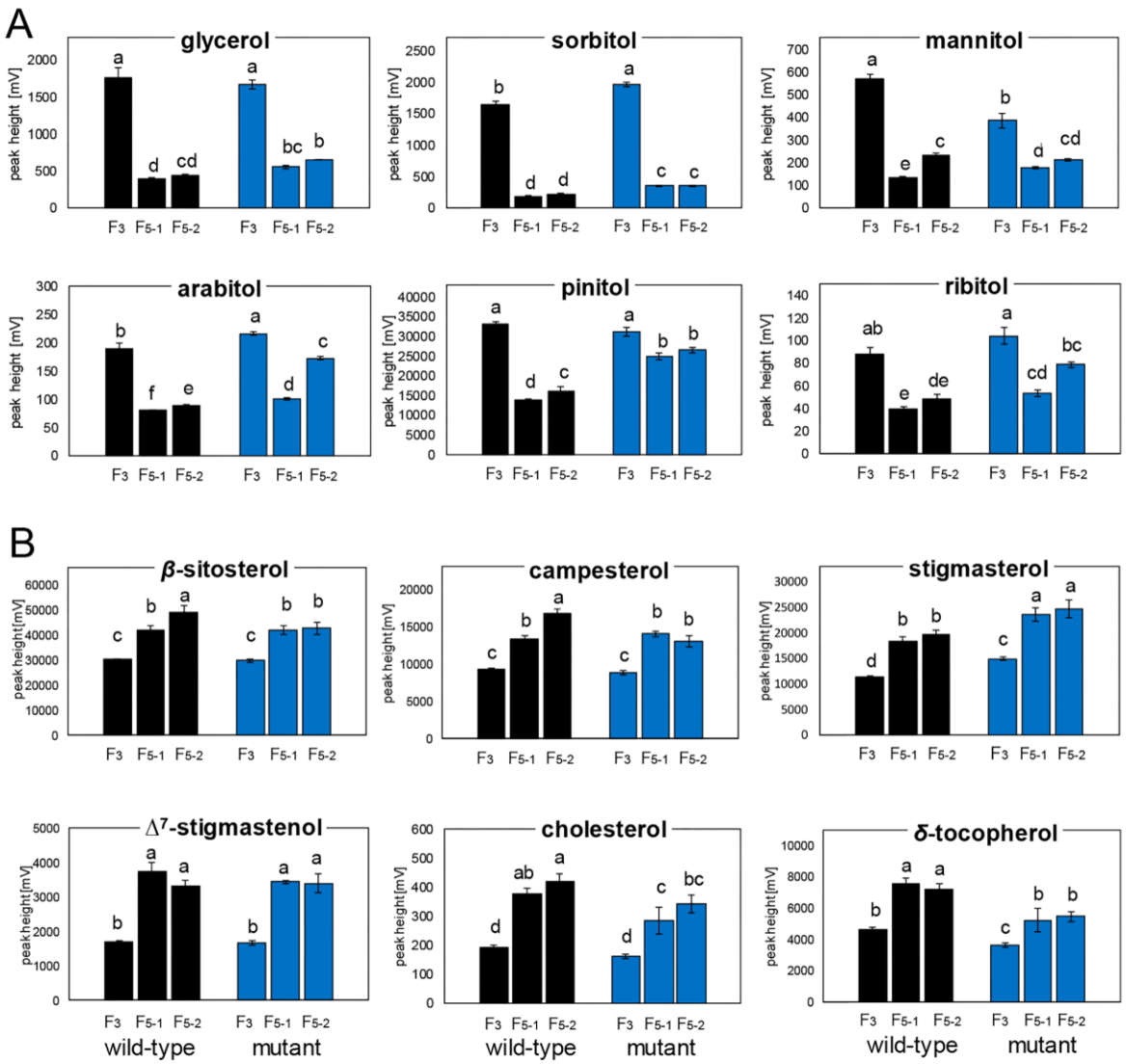

Figure 4. Statistically significant changes in sugar alcohols (A) and sterols (B) between F_3 and F_5 progenies observed for both homozygous wild-types (black) and homozygous *lpa* mutants (blue). For each metabolite, different letters indicate statistically significant differences in relative peak intensities (ANOVA with multiple pairwise comparisons by Tukey's post-hoc test or Kruskal–Wallis with Conover–Iman post-hoc test and Bonferroni correction, or Welch-ANOVA with Games-Howell post-hoc test; $p < 0.05$, respectively).

acid and have previously been reported to be significantly decreased in the *MIPS1* soybean mutant TW-*lpa*.[17] Therefore, their contents in the crossing parents TW-1-M-*lpa* and ZXD as well as in the homozygous wild-type and *lpa* mutant F_3 and F_5 progenies were quantitated based on relative peak intensities and are summarized in the pathway shown in Figure 3.

Besides the intended decrease of the content of phytic acid, which was determined via HPIC,[22] the metabolic differences between the employed *lpa* mutant (TW-1-M-*lpa*) and the wild-type crossing partner (ZXD) were characterized by significantly decreased contents of RFOs, i.e., raffinose and stachyose, the galactosyl cyclitols galactopinitol A, galactopinitol B, and ciceritol. Comparable differences in a similar range were also shown between the homozygous *lpa* mutant and the wild-type F_3 and F_5 progenies, grown in different seasons.

Metabolic Differences in Progenies from Different Generations and Growing Seasons. To explain the shift between F_3 and F_5 progenies observed in the PCA score plots (Figure 2), loading plots (Figures S2–S4) were employed and univariate comparisons of individual peaks were performed. In particular, for sugar alcohols and phytosterols, statistically significant changes between F_3 and F_5 progenies were observed for both homozygous wild-type and *lpa* mutant progenies (Figure 4). F_3 progenies, which were planted in spring 2014, showed higher contents of sugar alcohols (glycerol, sorbitol, mannitol, arabitol, pinitol, ribitol) and lower contents of phytosterols (*β*-sitosterol, campesterol, stigmasterol) than those of F_5, grown in autumn 2015.

Separation of Wild-Types and *lpa* Mutants via Supervised Statistical Assessment. As a further tool, OPLS-DA was applied for the metabolite profiling data of the

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combined fractions to identify metabolites differentiating wild-type and *lpa* mutant soybean seeds. The score plot of the OPLS-DA model is presented in Figure 5. A clear differentiation of

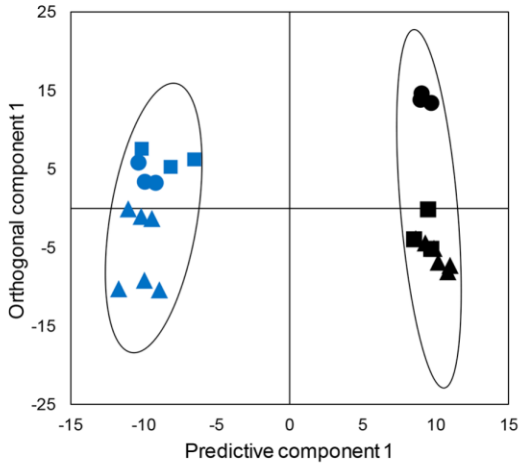

Figure 5. OPLS-DA score plot discriminating homozygous wild-type (black) and homozygous *lpa* mutant (blue) soybean seeds based on metabolite profiling data of the combined fractions: TW-1-M-*lpa* and WT crossing parent ZXD (circles), homozygous F_3 (squares), and F_5 (triangles) progenies. Drawn ellipses indicate 95% confidence limits based on the Hotelling's T2 hypothesis.

wild-type and *lpa* mutants was observable on the predictive axis. The overall quality of fit (R^2Y) was 0.908 and the overall cross-validation (Q^2Y) 0.868, which demonstrated a good reliability of the fitting and predictive ability of the OPLS-DA model. Permutation tests (n = 2000) also showed that the model was robust without overfitting ($p < 0.0005$).

Metabolites exhibiting VIP values ≥ 1.5 were regarded as discriminatory metabolites that dominated the separation between wild-type and *lpa* mutants. They all showed statistically significant differences in their mean concentrations (expressed as relative peak intensities) for all wild-types and all *lpa* mutants (Table 1, Figure S5). These 38 metabolites included organic acids (e.g., citric acid, phosphoric acid), lipophilic compounds (e.g., C20:0 FAME, C18:2 FFA, farnesol), a broad range of amino acids (e.g., leucine, threonine, asparagine, methionine, phenylalanine, and histidine), and biogenic amines (e.g., GABA, *β*-alanine). Except for phenylalanine, all other amino acids and biogenic amines were significantly increased (up to 6.7 times) in *lpa* mutants compared to wild types. In contrast, the previously described RFOs (e.g., raffinose, stachyose) and galactosyl cyclitols (e.g., galactopinitol A and B) showed pronounced decreases in *lpa* mutants, resulting in contents up to 96% lower than in wild-type soybean seeds.

DISCUSSION

The intended mutation-induced trait, i.e., reduced phytic acid contents compared to the original wild-type, was not affected by cross-breeding of the *lpa* mutant TW-1-M-*lpa* with a commercial cultivar. The $InsP_6$ contents determined for TW-1-M-*lpa* and the homozygous *lpa* F_3 and F_5 progenies (approximately 10 mg/g dry matter, Figure 3) were in a similar range as those previously described for *lpa* mutant soybean seeds.[17,26] A recent study has demonstrated that cross and selection breeding of TW-1-M-*lpa* with ZXD also did not change the distributions of lower inositol phosphate isomers ($Ins(1,2,3,4,5)P_5$ and $Ins(1,2,4,5,6)P_5$) in the resulting homozygous *lpa* mutant progenies.[22]

In eukaryotic cells, phytic acid can be formed via lipid-dependent and lipid-independent pathways. For the latter, the biosynthesis starts with the conversion of D-glucose 6-phosphate to 1D-*myo*-inositol 3-phosphate ($Ins(3)P_1$) through the enzyme 1D-*myo*-inositol 3-phosphate synthase (*MIPS1*) followed by stepwise ATP-dependent phosphorylation by specific inositol phosphate kinases (*InsPK*) (Figure 3).[27,28] Besides $Ins(3)P_1$, *myo*-inositol can also be utilized for the consecutive phosphorylation to form phytic acid. The lipid-dependent pathways result from the hydrolysis of $PtdIns(4,5)P_2$ by Phospholipase C (*PLC*) and subsequent phosphorylation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) by *InsPKs*.[29] *Myo*-inositol is not only the initial substrate of $InsP_6$ synthesis but also a central precursor for several metabolic processes, and its phosphorylated derivatives are of importance to signal transduction.[30] This is reflected in consistent metabolic changes in the concentrations of several RFOs and galactosyl cyclitols in the homozygous *MIPS1* mutants (Figure 3) which are in agreement with those previously described for the primary mutant TW-*lpa*.[16,17] The markedly decreased concentrations of *myo*-inositol, raffinose, stachyose, galactopinitol A, galactopinitol B, and ciceritol in homozygous *lpa* mutant progenies remained nearly unaffected by cross-breeding with a commercial cultivar. The fluctuation of metabolite levels in progenies from different generations and growing seasons, respectively, did not compromise the mutation-induced metabolic differences between homozygous wild-type and *lpa* mutant progenies. For sucrose, higher concentrations in the *lpa* mutant TW-*lpa* compared to its respective wild-type Taiwan 75 have been described;[16,17] this effect could be confirmed in the F_3 and F_5 progenies obtained by crossing TW-1-M-*lpa* and the commercial cultivar ZXD (Figure 3). The unexpectedly high content of sucrose in ZXD did not impair this *MIPS1* mutation-specific metabolic difference between homozygous wild-type and *lpa* mutant progenies.

Humans and nonruminant animals show a deficiency of *α*-galactosidase in the intestinal mucosa; therefore, they cannot digest RFOs.[31] As a consequence, microbial fermentation of RFOs in the distal digestive tract results in flatulence, diarrhea, and reduced feed efficiency.[32,33] The *lpa* mutants carrying the *MIPS1* mutation target analyzed in this study combine the advantages of a reduced phytic acid content with decreased RFOs levels. The fact that this nutritionally highly attractive compositional feature is also expressed in homozygous *lpa* progenies obtained by crossing the *lpa* mutant with a commercial cultivar is very promising from a breeder's point of view.

Besides these effects on sugar-based metabolites, the homozygous *lpa* *MIPS1* mutants showed consistent and remarkable changes in the contents of various free amino acids and biogenic amines (Table 1). Statistically significant increases of valine, leucine, serine, *β*-alanine, asparagine, citrulline, and tyrosine have been reported in the primary *lpa* mutant TW-*lpa* compared to the wild-type Taiwan 75.[17] Except for tyrosine, the increased concentrations of these free amino acids in the *lpa* mutant progenies could be confirmed in this study. Differences in tyrosine levels were superimposed by changes depending on generations (F_3 vs F_5) and/or growing seasons (spring 2014 vs autumn 2016) (Table S6). Moreover, the responses of a number of other amino acids were shown to be significantly increased in *lpa* mutant progenies (Table 1), e.g., glycine, histidine,

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Table 1. Metabolites Contributing to the OPLS-DA Separation of Wild-Type (WT) and *Lpa* Mutant Soybean Seeds (VIP > 1.5) from the Cross TW-1-M-*lpa* x ZXD

		response[a]			
metabolite	VIP	WT	*lpa*	ratio	*p* value
phosphoric acid	2.15	208 ± 50	1163 ± 337	5.60	<0.0001[c]
raffinose	2.22	346.3 ± 35.5	134.9 ± 11.6	0.39	<0.0001[b]
stachyose	2.21	280.7 ± 60.1	11.4 ± 2.4	0.04	<0.0001[c]
galactopinitol A	2.07	50.6 ± 19.9	6.2 ± 1.9	0.12	<0.0001[c]
galactopinitol B	2.14	56.0 ± 17.2	7.0 ± 1.8	0.13	<0.0001[c]
myo-inositol	1.92	22.5 ± 8.5	5.4 ± 3.7	0.24	<0.0001[c]
ciceritol	1.94	7.0 ± 3.7	1.0 ± 0.8	0.14	<0.0001[d]
glycine	1.72	19.4 ± 4.4	74.6 ± 49.6	3.85	<0.0001[d]
alanine	1.96	129 ± 23	587 ± 289	4.56	<0.0001[d]
valine	1.53	19.8 ± 1.6	29.5 ± 7.3	1.49	0.0008[c]
leucine	1.68	20.9 ± 5.2	35.9 ± 8.1	1.71	<0.0001[c]
serine	1.80	12.1 ± 1.9	61.3 ± 43.5	5.06	<0.0001[d]
homoserine	1.51	1.6 ± 0.7	3.6 ± 1.7	2.29	0.0016[c]
threonine	1.99	9.2 ± 0.9	26.9 ± 10.1	2.93	<0.0001[d]
methionine	1.73	5.4 ± 1.3	12.0 ± 4.8	2.21	0.0006[c]
phenylalanine	1.56	26.4 ± 6.8	17.8 ± 3.1	0.67	0.0011[c]
tryptophan	1.70	44.9 ± 6.3	80.3 ± 22.6	1.79	0.0002[c]
histidine	1.80	16.2 ± 5.0	76.8 ± 49.2	4.74	<0.0001[d]
asparagine	1.69	5.5 ± 5.9	35.6 ± 20.0	6.51	0.0002[c]
β-alanine	1.97	19.3 ± 9.5	129.3 ± 73.5	6.68	<0.0001[d]
citrulline	1.92	2.6 ± 0.9	5.9 ± 1.3	2.24	<0.0001[b]
2-aminobutyric acid	1.77	0.8 ± 0.4	2.8 ± 1.3	3.52	<0.0001[b]
γ-aminobutyric acid	2.11	20.8 ± 5.1	114.9 ± 38.8	5.52	<0.0001[c]
citric acid	1.66	1142 ± 143	830 ± 139	0.73	<0.0001[b]
syringic acid	1.75	11.6 ± 2.0	16.3 ± 2.4	1.40	<0.0001[c]
mucic acid	1.54	3.4 ± 2.3	5.7 ± 1.7	1.66	0.0080[d]
3-glycerophosphoric acid	1.91	3.4 ± 0.5	8.7 ± 2.9	2.60	<0.0001[c]
C20:0 FAME	1.51	400 ± 48	480 ± 46	1.20	0.0004[b]
C24:0 FAME	1.78	251 ± 44	379 ± 59	1.51	<0.0001[b]
C26:0 FAME	2.01	24.5 ± 4.7	46.4 ± 7.1	1.89	<0.0001[b]
C17:1 FAME	1.67	86.0 ± 14.2	119 ± 16	1.38	<0.0001[b]
C20:1 FAME	1.60	393 ± 104	597 ± 87	1.52	<0.0001[b]
C22:1 FAME	1.63	7.1 ± 2.0	12.2 ± 3.1	1.72	<0.0001[b]
C20:2 FAME	2.02	50.3 ± 4.4	28.8 ± 5.6	0.57	<0.0001[b]
C18:2 FFA	1.73	2452 ± 295	3339 ± 578	1.36	0.0001[b]
farnesol	1.63	42.5 ± 15.2	86.1 ± 27.0	2.03	<0.0001[c]
phytol	1.55	43.5 ± 11.8	69.0 ± 17.1	1.59	0.0003[b]

[a]All values are expressed as relative peak intensities, i.e., metabolite peak intensity/(internal standard peak intensity/100). For each metabolite, the response of the WT was the mean value of ZXD and the homozygous wild-type F_3 and F_5 progenies (three aliquots per generation); the response of the *lpa* mutant was the mean value of TW-1-M-*lpa* and the homozygous *lpa* mutant F_3 and F_5 progenies (three aliquots per generation). [b]Student's *t*-test [c]Welch's *t*-test [d]Mann−Whitney test

methionine, and tryptophan. It is noteworthy that significantly increased amounts of free amino acids have been observed not only in the primary *MIPS1 lpa* mutant grown under various growing conditions[17] but also in homozygous *lpa* progenies of different generations obtained after crossing of a *MIPS1 lpa* mutant with a commercial cultivar. Considering that these progenies were grown under conditions different from those previously reported and that they did not have background mutation effects,[17,34] the newly elaborated data strongly support that these changes in the concentrations of free amino acids are the result of pleiotropic *MIPS1* effects and not caused by environmental impact. However, there is presently no obvious explanation of this phenomenon on a molecular level.

Two individual homozygous wild-type and *lpa* mutant F_5 progenies, which had been obtained from individual heterozygous plants grown in the same season, were investigated. Considerable differences between individuals were observable for several metabolites, both in homozygous wild-type and in *lpa* mutant progenies (Figures 3 and 4, Tables S5 and S6); this may be explained by the genetic heterogeneity among different plants.[35] The differentiation between individuals in the PCA score plots (Figure 2) was particularly pronounced among *lpa* mutant progenies, which also reflects the genetic heterogeneity among plants of segregating populations.

Furthermore, metabolite changes were analyzed in homozygous WT and *lpa* mutant progenies of different generations. The F_3 and F_5 seeds were grown in the same experimental field but in different seasons (F_3, from April to July 2014; F_5, from August to October 2015). Accordingly, metabolic differences may be attributable to both genetic effects, resulting from the random selection of segregating populations, and to environmental impact. Univariate analyses of the metabolite profiling

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data revealed significant differences in soybean seeds' concentrations of sterols and sugar alcohols both in homozygous *lpa* mutant and wild-type lines. F_5 progenies exhibited significantly lower concentrations of sugar alcohols (on average −57%) and significantly higher contents of sterols like β-sitosterol, campesterol, stigmasterol, cholesterol, and Δ^7-stigmastenol (on average +75%) compared to F_3 progenies (Figure 4, Table S6). Sugar alcohols are known to contribute to the adjustment of plants during drought stress. During water deficiency, sugar alcohols and other osmotically active compounds are accumulated to balance the osmotic difference between the cell's surroundings and the cytosol.[36,37] Phytosterols have various biological functions in plants. They act as precursors for brassinosteroids, regulate the fluidity of membranes, and are therefore likely to be of importance to the adaption of membranes to temperatures.[38] Decreased phytosterol contents have both been described for rye grown under dry and warm climatic conditions[39] and in drought-stressed barley.[40] The consistent differences in sugar alcohols and sterols between F_3 and F_5 progenies observed in the present investigation correlate with the different climatic conditions (rising vs falling temperature profile; patterns of precipitation) in the respective growing periods (Figure S6).

Yuan et al. (2007) reported a reduced field emergence rate for the primary mutant TW-*lpa* compared to its respective wild-type Taiwan 75, especially when planted in subtropical climates.[11] TW-1-M-*lpa*, the soybean mutant employed as a crossing parent in this study, has been reported to have a significantly higher seed field emergence rate and better germination traits than TW-*lpa*.[15] Multiple backcrossing experiments of *lpa* soybean lines with normal-phytate lines have been shown to be useful for the generation of *lpa* lines with normal field emergence[18] and to achieve yields in the *lpa* progenies statistically equivalent to high-yielding soybean cultivars.[41] In the present study, data on the agronomic performance of progenies resulting from the cross-breeding experiment could not be evaluated due to the low amount of sample materials. Larger field experiments would be required for valid conclusions on the agronomic performance of the generated *lpa* mutant progenies.

Despite the described fluctuations in metabolites depending on generations and/or growing seasons, the supervised statistical assessment OPLS-DA showed a clear separation of the wild-type and the *lpa* mutant soybean seeds and allowed the identification of various discriminatory metabolites (Table 1). Cross-breeding of the *lpa* soybean mutant TW-1-M-*lpa* with the commercial cultivar ZXD did not influence the metabolic phenotype previously described for the primary mutant[17] in the resulting homozygous *lpa* progenies. The metabolite profiling data showed that the employed mutation breeding resulted in the pursued *lpa* trait, i.e., the reduction of phytic acid content. In addition, it also led to consistent and desirable alterations in the concentrations of other nutritionally relevant soybean constituents, namely, sucrose and RFOs, the most important soluble carbohydrates in mature soybean seeds,[42] and stable increases in the amounts of various free amino acids, among them several essential ones. The observed consistency and stability of these metabolic changes in progenies over several generations is a major prerequisite for the integration of these mutants into commercial breeding programs. For *lpa* rice, a recent study also demonstrated that the complex metabolic alterations as a result of the *OsSULTR3;3* mutation were nearly unaffected by cross-breeding of the *lpa* mutant.[19] However, it should be kept in mind that these results cannot be directly transferred to other *lpa* mutation types or crossing parents. Furthermore, cross-breeding of the *lpa* soybean mutants with other commercial, high-performance cultivars might be useful to provide a substantiated insight into the general stability and consistency of metabolic changes of the *MIPS1* mutation.

Nevertheless, the data indicate that the *MIPS1 lpa* mutant as well as its progenies might be valuable genetic resources for commercial breeding programs to produce soybean seeds stably exhibiting improved phytate-related traits, e.g., mineral bioavailability and reduced environmental phosphorus pollution, as well as nutrition-related features, e.g., metabolizable energy and carbohydrate digestibility.[33,43]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b00817.

Chromatographic and mass spectral data of identified compounds; overview of compounds significantly and consistently altered by the *MIPS1* mutation (OPLS-DA VIP > 1.5) or by different generations and growing seasons; genotyping results; PCA loading plots of the combined and single fractions; S-plot of the OPLS-DA model; meteorological data (DOCX)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

24-MCA, 24-methylene-cylcoartanol; ANOVA, analysis of variance; FAME, fatty acid methyl ester; FFA, free fatty acid; FID, flame ionization detector; GABA, γ-aminobutyric acid; GC, gas chromatography; HPIC, high pressure ion chromatography; $InsP_6$, phytic acid; LDPE, low-density polyethylene; *lpa*, *low phytic acid*; *MIK*, *myo*-inositol kinase; *IMP*, *myo*-inositol monophosphatase; *MIPS1*, D-*myo*-inositol 3-phosphate synthase gene 1; MS, mass spectrometry; OPLS-DA, orthogonal partial least-squares-discriminant analysis; PC, principal component; PCA, principal component analysis; RFOs, raffinose family oligosaccharides; SPE, solid phase extraction; UV, ultraviolet; VIP, variable importance in projection; WT, wild-type.

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4 DISCUSSION

4.1 Methodology

The objectives of the studies underlying this dissertation were to investigate the impact of cross-breeding of *lpa* soybean mutants with commercial wild-type cultivars on (i) the contents of phytic acid and lower inositol phosphate isomers, and (ii) the mutation-induced metabolite signature in the resulting homozygous wild-type and homozygous *lpa* mutant progenies. Aspects regarding the respective study design, the applied methodologies, the interpretation of the elaborated metabolite profiling and inositol phosphate data and their potential nutritional significance will be discussed in the following sections.

Determination of phytic acid and lower inositol phosphates

The first methods for the determination of phytic acid involved the non-specific formation of precipitates with Fe^{3+} and quantifications on the basis of the iron (Heubner and Stadler, 1914) or the phosphorus content of the resulting precipitates (McCance and Widdowson, 1935). A major drawback of these non-specific methods was their low sensitivity (Skoglund and Sandberg, 2002). Ion-pair reversed-phase HPLC enabled the simultaneous separation and detection of phytic acid and other lower inositol phosphates; however, a differentiation of isomeric forms was not possible (Lehrfeld, 1994; Sandberg and Ahderinne, 1986). These methods were based on the application of tetrabutylammonium hydroxide as ion-pair agent in the mobile phase (Sandberg and Ahderinne, 1986), which is able to bind the negatively charged inositol phosphates and to interact with the non-polar HPLC stationary phase. Besides such chromatographic approaches, spectroscopic methods have also been used to quantify phytic acid and lower inositol phosphates (Schlemmer *et al.*, 2009). NMR is able to determine stereoisomers which often co-eluate during chromatographic separation (Phillippy, 1989) and offers high accuracy and specificity (Duong et al., 2018). However, the main disadvantages of this technique are the expensive equipment and its low sensitivity making NMR unsuitable for the detection of low concentrations of inositol phosphates (Skoglund and Sandberg, 2002), as they are often found in biological samples. In contrast, high pressure ion chromatography (HPIC) methods offer good reproducibility and sensitivity, even allowing the discrimination between isomeric forms of inositol

phosphates at low concentrations (Skoglund and Sandberg, 2002). These properties make HPIC nowadays one of the most commonly used approaches for the simultaneous determination of phytic acid and lower inositol phosphate isomers (Blaabjerg *et al.*, 2010).

Therefore, in the present study an HPIC-based isomer-specific analytical technique, which has originally been developed for a corn-based animal feed (dried distillers grains with solubles) (Oates *et al.*), was adapted to the soybean matrix. Inositol phosphates were separated with an acidic eluent system and detected via ultraviolet absorbance at 290 nm after post-column derivatization with $Fe(NO_3)_3$. There is currently no procedure available which allows the complete separation and simultaneous measurement of all inositol phosphates, taking into consideration their stereoisomers (Duong *et al.*, 2018). However, the HPIC approach employed in this study allowed the detection of in total 25 peaks representing 32 *myo*-inositol phosphate isomers (6 $InsP_2$, 12 $InsP_3$, 9 $InsP_4$, 4 $InsP_5$ and $InsP_6$), without separation of enantiomers (Chen and Li, 2003; Oates *et al.*). The order of elution of the InsP isomers correlated with the increasing number of phosphate groups. Furthermore, only compounds which react with $Fe(NO_3)_3$ during post-column derivatization and have an absorbance near 290 nm are detected with the applied method, resulting in a very good selectivity compared with e.g. conductivity detection (Oates *et al.*). The validation of the method proved the good repeatabilities (2.6% RSD for 50 mg; 1.1% RSD for 400 mg), reproducibilities (4.1% RSD for 50 mg; 3.1% RSD for 400 mg) and recoveries (102% for 50 mg; 96% for 400 mg), with LODs and LOQs in the range of 0.5-1.2 µg/mL and 1.4-3.6 µg/mL injection volume, respectively. Duo to the low abundance of $InsP_2$ isomers in soybean seeds, they were detectable but not quantifiable with the applied procedure.

The number and position of the phosphorylations of the *myo*-inositol ring determine the distinct biological properties and physiological functions of the inositol phosphates (Schlemmer *et al.*, 2001). However, the properties or functions may differ for opposite enantiomers. For example, Ins(1,4,5,6)P_4 can completely inhibit the response of IGF-1, whereas its enantiomer Ins(3,4,5,6)P_4 only shows 25% inhibition (Razzini *et al.*, 2000); on the other hand, Ins(3,4,5,6)P_4 is an inhibitor of the calcium-mediated chloride secretion (Ismailov *et al.*, 1996). Furthermore, Ins(1,4,5,6)P_4 originates from the lipid-dependent pathway, whereas Ins(3,4,5,6)P_4 is synthesized lipid-independently (Raboy, 2003). Unfortunately, the separation of enantiomers was not possible with the

applied HPIC methodology. Due to this drawback, the results obtained in the present study at some points did not allow to draw final conclusions and interpretations about both the biosynthesis and the biological function of the described inositol phosphate isomers in soybean seeds.

Besides the non-separation of enantiomers, the fact that several of the inositol phosphate isomers were not commercially available, hampered their unambiguous identification. It has been demonstrated that in such cases, inositol phosphate isomers can be obtained by specific hydrolyses (Chen and Li, 2003). In this study, some of the identifications were also based on data resulting from this strategy.

Metabolite profiling

The GC-based metabolite profiling approach applied in this study is based on a method originally developed for rice seeds (Frenzel *et al.*, 2002), which has been adapted to various other matrices such as soybean (Frank *et al.*, 2009), maize (Röhlig and Engel, 2010), mung bean (Na Jom *et al.*, 2011), and barley (Lanzinger *et al.*, 2015). All these approaches comprise the consecutive extraction of lipophilic and polar constituents with solvents of different polarities. Subsequently, fractionation steps including transesterification and solid phase extraction for lipophilic extracts as well as silylation and selective hydrolysis for polar extracts are used to separate major from minor constituents. The obtained four fractions contain a broad spectrum of low molecular weight (< 1000 Da) constituents including fatty acid methyl esters (FAMEs) and hydrocarbons (fraction I), free fatty acids (FFAs), fatty alcohols, tocopherols, triterpenic alcohols, and phytosterols (fraction II), sugars and sugar alcohols (fraction III), and amino acids, amines as well as organic and inorganic acids (fraction IV).

In addition, this method has been successfully used not only to study the differences between *lpa* and conventional crops (Frank *et al.*, 2007; Frank *et al.*, 2009) and the metabolic effects of GM maize (Frank *et al.*, 2012b), but also to investigate a broad range of topics such as the metabolic differences between maize originating from conventional vs. organic farming (Röhlig and Engel, 2010), the metabolic responses of barley to drought stress (Lanzinger *et al.*, 2015; Wenzel *et al.*, 2015), and the metabolic differences between colored rice samples (Frank *et al.*, 2012a). Furthermore, this GC-approach has been used to investigate the time-dependent

changes during germination of rice (Shu *et al.*, 2008), sprouting of mung bean (Na Jom *et al.*, 2011), and malting of barley (Frank *et al.*, 2011).

Several minor adaptions of the original methodology applied for rice have been introduced to improve its applicability to the soybean matrix, which is characterized by high protein and fat contents compared to rice (Liu, 1997a). Frank *et al.* (2009b) used 300 mg soybean flour instead of 2 g rice flour to extract low molecular weight constituents and added 200 mL methanol for cell digestion. The applied amounts for the internal standard solutions were adjusted to 100 µL of internal standards I and II, and to 250 µL of internal standards III and IV, respectively. Before the silylation of fraction II, 100 µL of retention time mix I were added. Before the fractionation, the cloudy polar extract was passed through a 0.45 µm PTFE filter. For fractions III and IV, 1 mL and 2 mL of the filtrated polar extract were evaporated to dryness under vacuum, respectively. After silylation of fraction III, 300 µL of *n*-hexane and 300 µL of deionized water were added for selective hydrolysis. The mixture was shaken and after phase separation, 100 µL of the upper phase, containing sugars and sugar alcohols, were mixed with 50 µL of retention time standard I. The GC analysis time for fraction III needed to be expanded from 65 min to 80 min to allow the detection of the late-eluting tetrasaccharide stachyose. In the present study, the following additional modifications of the method described by Frank *et al.* (2009b) were introduced to improve the reproducibility of the results: The silylation times of fractions III and IV were increased from 15 min to 20 min at 70 °C. Furthermore, the cloudy extract of fraction IV was subjected to an additional 0.45 µm PTFE filtration step before GC-based analysis. To quantitate highly concentrated constituents of fraction I, such as C18:2 FAME, a 1:10 dilution (v/v) was applied.

Even though the applied fractionation approach is time-consuming and thereby not suitable for a high-throughput screening, it offers good reproducibility and a broad area of possible applications. Due to the high complexity of the metabolome, which has been estimated to comprise up to 20,000 metabolites in a single plant (Fernie *et al.*, 2004), the fractionation steps offer the potential to determine a broader spectrum of (minor) metabolites which otherwise might have been overlapped by constituents from other compound classes. Such pre-fractionation steps and the use of different analytical techniques have been recommended for metabolomics experiments to obtain a more comprehensive snapshot of the metabolome (Goodacre *et al.*, 2004; Sumner *et al.*, 2002). Complementary analytical techniques like LC-MS or NMR allow

the detection of thermo-labile and high-molecular weight metabolites without the requirement for derivatization. Thereby, a broad array of secondary metabolites including flavonoids, vitamins or phospholipids could be investigated. Secondary metabolites might have also been modulated by the *lpa* mutation event or the cross-breeding step and play an important role in the final evaluation of the nutritional quality of the investigated soybean seeds.

Statistical assessment

To extract the relevant information from the huge and complex raw data sets of metabolomics experiments, suitable statistical methods including multivariate and univariate approaches are essential (Korman *et al.*, 2012). In addition, pre-processing and pre-treatment steps are of fundamental importance to enhance the biological interpretability of the metabolomics data (van den Berg *et al.*, 2006). Therefore, before the metabolite profiling data were used for multivariate analysis, different types of data transformations (log transformation, cube root transformation) and data scaling (mean centering, auto, pareto and range scaling) as well as their combinations were tested (Chong *et al.*, 2018). For the elaborated dataset, cube root transformation in combination with pareto scaling was shown to be the most suitable approach to approximate the normal distribution.

In the next step, the unsupervised principle component analysis (PCA) was used to gain a first overview on the distribution of the data points. PCA loading plots were employed to identify metabolites contributing to the discrimination trend observed in the score plots. As a next step, partial least square discriminant analysis (PLS-DA) was employed to the metabolite profiling data as a supervised multivariate statistical approach for the differentiation of wild-type and *lpa* mutant soybean seeds. However, due to the variation between different generations and/or growing locations within these groups, the PLS-DA model was shown to be over-fitted. Therefore, the orthogonal partial least squares-discriminate analysis (OPLS-DA) was used, an alternative supervised method which is suitable for the classification of data with multi-collinear and noisy variables. For this approach, samples were classified into pre-defined groups, i.e. wild-types and *lpa* mutants, the data were filtered using orthogonal signal correction (OSC) into group-predictive and group-unrelated (orthogonal) components to achieve the best prediction of group membership (Bylesjö *et al.*, 2006).

Permutation tests and cross-validations proved that the OPLS-DA model was reliable without over-fitting. Together with the S-plot and variable importance in projection (VIP) values, the OPLS-DA model allowed the visualization and identification of the individual metabolites contributing to the differentiation between wild-type and *lpa* mutant soybean seeds. The discriminatory metabolites were subsequently analyzed by means of univariate analyses and provided useful information for creating a deeper understanding of the biological network in *lpa* soybean mutants.

4.2 Selection of crossing partners

Lpa mutants

During the past years, several *lpa* soybean mutants have been developed using chemical (Hitz *et al.*, 2002; Wilcox *et al.*, 2000) and physical mutagenesis (Yuan *et al.*, 2007) as well as genetic engineering via transformation (Bilyeu *et al.*, 2008) or gene silencing (Nunes *et al.*, 2006; Punjabi *et al.*, 2018). In general, such mutations can impair (i) the function of enzymes involved in the substrate supply pathway for the biosynthesis of phytic acid, i.e. the formation of inositol or Ins(3)P_1, (ii) the function of enzymes involved at the end of the pathway leading to phytic acid, or (iii) the compartmentation, transportation, or storage of phytic acid to the vacuole (Sparvoli and Cominelli, 2015). In the present investigation, the three *lpa* soybean mutant lines *Gm-lpa*-TW-1 (TW-*lpa*), *Gm-lpa*-TW-1-M (TW-1-M-*lpa*) and *Gm-lpa*-ZC-2 (ZC-*lpa*), which had been obtained from the wild-type cultivars Taiwan 75 and Zhechun No. 3 (ZC-3), respectively, via 150 Gy ^{60}Co *γ*-irradiation (Yuan *et al.*, 2007), were used for cross-breeding experiments with commercial wild-type cultivars. Molecular characterization revealed that the phytic acid reductions in TW-*lpa* and TW-1-M-*lpa* resulted from a 2 bp deletion in the D-*myo*-inositol 3-phosphate synthase gene 1 (*MIPS1*) (Yuan *et al.*, 2007; Yuan *et al.*, 2017). The enzyme D-*myo*-inositol 3-phosphate synthase (MIPS, EC 5.5.1.4) catalyzes the NADH-dependent conversion of D-glucose 6-phosphate to *myo*-inositol 3-phosphate and is thereby the first and rate-limiting step in the biosynthesis of both *myo*-inositol and phytic acid (Bhati *et al.*, 2016; Kumari *et al.*, 2012; Loewus and Murthy, 2000). Four highly conserved *MIPS* gene isoforms (*GmMIPS1-4*) have been identified in soybean (Hegeman *et al.*, 2001) with *MIPS1* being the major isoform with high expression levels in developing seed tissues (Kumari *et al.*, 2012). Therefore, *MIPS1* has been regarded as a suitable target to

generate soybean lines with reduced phytic acid contents in the seeds without influencing the vital aspects of inositol metabolism in other plant tissues (Kumari *et al.*, 2012). The phytic acid reduction of about 53% in TW-*lpa* compared to its WT precursor Taiwan 75 was shown to be accompanied by a broad range of changes of other nutritionally relevant metabolites including decreased contents of *myo*-inositol, raffinose, stachyose, and the galactosyl cyclitols galactopinitol A and B as well as increased concentrations of sucrose and various free amino acids, but without an accumulation of lower inositol phosphates in TW-*lpa* (Frank *et al.*, 2009). In comparison to TW-*lpa*, its natural mutant TW-1-M-*lpa* possessed higher germination speed (96 h vs. 72 h) and germination percentage both during warm (85% vs. 80%) and accelerated aging (80% vs. 45%) germination tests, as well as a higher rate of seed field emergence (50% vs. less than 10%) after a 2-year storage at room temperature (Yuan *et al.*, 2017). Therefore, the *MIPS1* mutant TW-1-M-*lpa* was considered to be particularly suitable for studying the impact of cross-breeding with a commercial wild-type cultivar on the stability of the *MIPS1* mutation-induced metabolite signature in homozygous *lpa* mutant progenies by means of metabolite profiling.

In contrast, the phytic acid reduction of about 46% in ZC-*lpa* compared to its wild-type progenitor ZC-3 resulted from a G → A mutation in chromosome 14 of the inositol 1,3,4,5,6-penta*kis*phosphate 2-kinase (*GmIPK1*) (Yuan *et al.*, 2012). The enzyme inositol 1,3,4,5,6-penta*kis*phosphate 2-kinase (IPK1, EC 2.7.1.158) catalyzes the last step in the biosynthesis of phytic acid by phosphorylating Ins(1,3,4,5,6)P_5 in the second position to form InsP_6. Two additional homologous genes of *GmIPK1* (*Glyma04g03240* and *Glyma06g03310*) have been identified in soybean, but in contrast to *IPK1*, their expression levels are rather low (Yuan *et al.*, 2012). Comparative investigations by Frank *et al.* (2009) showed that the *IPK1* mutation resulted in significant accumulations of InsP_3, InsP_4 and InsP_5, but without noteworthy additional metabolic changes. However, during this stage, no data on the stereoisomers of these accumulated lower inositol phosphates were available. Taking these facts into consideration, the *IPK1* mutant ZC-*lpa* was considered as an attractive candidate to investigate the impact of cross-breeding with commercial wild-type cultivars on the intended effect of phytic acid reduction and the occurrence of lower inositol phosphate isomers in the resulting progenies.

Wild-types

The commercial soybean cultivars Zhexiandou No. 4 (ZXD) and Cu as well as Zhechun No. 3 (ZC-3), the progenitor of ZC-*lpa* (Yuan *et al.*, 2007), were used as wild-type crossing parents in this investigation. All applied wild-type cultivars showed good germination rates (≥85%) and dry matter yields of 2−2.25 tons/ha, but differed in their compositional characteristics and origins. ZXD, which had been developed by Zhejiang Academy of Agricultural Sciences, is widely adapted to various Chinese provinces (Zhu *et al.*, 2010) and possesses high contents of sucrose and of total water-soluble sugars (TWSS) (Yu *et al.*, 2016). ZC-3 is characterized by its high protein content and is widely grown as a spring season soybean variety in Zhejiang and other provinces of southern China (Yuan *et al.*, 2009). The soybean variety Cu had been introduced from Australia and originates from wild soybean; it exhibits characteristic compositional features such as high fructose and glucose contents, but low sucrose, TWSS, and water-soluble protein (WSP) concentrations (Yu *et al.*, 2016).

In total, progenies from the following four crosses were obtained: (i) TW-1-M-*lpa* x ZXD; (ii) ZC-*lpa* x ZC-3; (iii) ZC-*lpa* x Cu; and (iv) ZC-*lpa* x TW-1-M-*lpa.*

In general, only few data on the inositol phosphate isomers present in soybean seeds are available (Blaabjerg *et al.*, 2010; Phillippy and Bland, 1988; Sun and Jaisi, 2018). Furthermore, the effects of crossing *lpa* soybean mutants with wild-type cultivars and the impact of *lpa* double mutants carrying two different mutation targets on the contents of inositol phosphate isomers were unknown. Therefore, the progenies of all generated crosses were analyzed by HPIC. In addition, the homozygous WT and homozygous *lpa* mutant progenies resulting from the cross TW-1-M-*lpa* x ZXD were characterized by means of metabolite profiling.

4.3 Nutritional significance of the elaborated data

Phytic acid and lower inositol phosphates

The integration of *lpa* soybean mutants into commercial cross- and selection-breeding programs requires the stability of the *lpa* trait, i.e. significantly reduced contents of phytic acid compared to the respective wild-type, upon cross-breeding. To verify this prerequisite, the *lpa* soybean mutants TW-1-M-*lpa* and ZC-*lpa* were crossed with WT cultivars or among themselves, and the inositol phosphate contents were investigated

via HPIC in homozygous progenies of several generations and from different growing seasons.

For both the *MIPS1* and *IPK1* mutation targets, the *lpa* trait in homozygous *lpa* mutant progenies was maintained upon cross-breeding, despite slight variations of absolute levels of phytic acid between different growing seasons/years. For the double *lpa* mutants, the reductions in phytic acid contents were particularly high and more pronounced than expected from the single mutants. The mean $InsP_6$ contents in homozygous *lpa* double mutant progenies were 3.60 mg/g, whereas mean contents of 9.63 mg/g were found for single *MIPS1* progenies and 8.98 mg/g for single *IPK1* progenies. For the *IPK1* mutant, crosses from two different WT crossing parents were obtained. Even though the phytic acid contents of ZC-3 and Cu did not significantly differ, crossing of ZC-*lpa* with its original WT precursor ZC-3 was shown to be more powerful to generate progenies with particularly low $InsP_6$ contents than crossing with the commercial WT Cu. This might be related to the different origins of these two WT cultivars; ZC-3 was introduced from China, whereas Cu originated from wild soybean of Australia (Yu *et al.*, 2016).

The consistently low contents of phytic acid in homozygous *lpa* mutant progenies are valuable from a nutritional and environmental point of view both for food and feed applications. Improved Ca and Zn utilization have been reported for *lpa* crops in animal studies, e.g. in rats (Poulsen *et al.*, 2001) and swine (Poulsen *et al.*, 2001; Spencer *et al.*, 2000). For humans, the *lpa* maize mutant *lpa*-1-1 with approximately 60% phytate reduction compared to the wild-type strain (Hambidge *et al.*, 2005), was shown to improve the Zn, Fe, and Ca absorption by 76% (Adams *et al.*, 2002), 49% (Mendoza *et al.*, 1998), and 43% (Hambidge *et al.*, 2005), respectively. In addition, various studies showed that *lpa* crops increase the availability of inorganic P in animals such as poultry (Ertl *et al.*, 1998; Li *et al.*, 2000), swine (Spencer *et al.*, 2000), and fish (Sugiura *et al.*, 1999). For example, experiments with pigs showed that GM *lpa* corn increases the bioavailability of P approximately five times compared to nearly isogenic equivalent normal-phytate corn hybrids, thereby reducing P excretion in manure and making the manure of *lpa* fed swine more environmentally and economically suitable for the application as fertilizer (Spencer *et al.*, 2000). Furthermore, *lpa* crops have been reported to improve the bone mineralization and growth performance of pigs (Spencer *et al.*, 2000; Veum *et al.*, 2001) and poultry (Li *et al.*, 2000; Li *et al.*, 2001). The improved growth performance of animals fed with *lpa* crops might be explained by the

inhibitory effect of $InsP_6$ on several digestive enzymes such as *α*-amylase, lipase, and different proteinases such as pepsin, trypsin and chymotrypsin (Denstadli *et al.*, 2006; Duong *et al.*, 2018; Khan and Ghosh, 2013; Liu *et al.*, 2010). Taking into consideration these advantages and the fact that about 98% of defatted soybean meal is employed as high protein source in animal feed, e.g. for poultry, pork, and aquaculture species (Goldsmith, 2008; Hartman *et al.*, 2011), the investigated *lpa* soybean mutants and their homozygous *lpa* progenies appear to be highly attractive for the animal feed industry.

In the following section, inositol phosphate isomers are numbered according to Table S2 from the Supporting Information of Publication I (see Chapter 3.1). The lower inositol phosphate isomer contents quantifiable in WT and *MIPS1 lpa* soybean seeds mainly corresponded to the isomers Ins(1,2,3,4,5)P_5/Ins(1,2,3,5,6)P_5 (**22**), Ins(1,2,4,5,6)P_5/Ins(2,3,4,5,6)P_5 (**23**), and Ins(1,3,4,5,6)P_5 (**24**). The *MIPS1* mutant TW-*lpa*, where the initial step in the biosynthesis of phytic acid is disrupted, has been reported to not accumulate lower inositol phosphate isomers compared to its WT progenitor Taiwan 75 (Frank *et al.*, 2009). The same applied for TW-1-M-*lpa*, another *MIPS1* mutant. After cross-breeding of TW-1-M-*lpa* with ZXD, the $InsP_5$ isomers were slightly increased in homozygous *lpa* mutant progenies compared to the respective wild-type progenies; however, their profiles and contents remained in a comparable order of magnitude. In contrast, the disruption of the last step in the biosynthesis leading to phytic acid by the *IPK1* mutation, resulted in significant accumulations of the lower inositol phosphate precursors of phytic acid, i. a. Ins(1,5,6)P_3/Ins(3,4,5)P_3 (**12**), Ins(4,5,6)P_3 (**13**), Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 (**20**), and Ins(1,3,4,5,6)P_5 (**24**) in soybean seeds, which could not be utilized to generate higher inositol phosphates. After crossing ZC-*lpa* with wild-type cultivars, the contents of lower inositol phosphate isomers were considerably increased. This effect was especially pronounced in the homozygous *lpa* mutant F_6 progenies resulting from the crossing of ZC-*lpa* with its WT progenitor ZC-3. Again, the observed differences in the accumulations of lower inositol phosphates between the two crosses might be explained by the different genetic backgrounds of the employed WT crossing parents ZC-3 and Cu. The double *lpa* mutants only showed moderate accumulations of the lower inositol phosphates Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 (**20**) and Ins(1,3,4,5,6)P_5 (**24**) due to the interruption of both the substrate supply pathway and the last step in the biosynthesis yielding phytic acid.

The mineral binding ability of inositol phosphates is known to be dependent on the number of phosphate groups per molecule: The less phosphate groups, the lower the binding capacity and stability of the formed phytate-mineral complexes *in vitro* (Persson *et al.*, 1998). Experiments with rats supported these findings and demonstrated that the Ca and Zn uptake was inhibited by $InsP_6$ and $InsP_5$, but not by $InsP_4$ and $InsP_3$ (Lönnerdal *et al.*, 1989). The Fe absorption of humans was also shown to be not perturbed by $InsP_4$ and $InsP_3$, if they were tested separately. However, applied as complex mixtures of various inositol phosphates, lower inositol phosphates were shown to be able to contribute to the inhibitory effects of mineral bioavailability, potentially via the formation of mineral complexes between different inositol phosphate isomers (Sandberg *et al.*, 1999). Therefore, it cannot be finally ruled out that the accumulations of lower inositol phosphate isomers in the *IPK1 lpa* mutants might to a minor degree counteract the beneficial effects of the reduced $InsP_6$ contents on the mineral bioavailability.

On the other hand, since the first description of Ins(1,4,5)P_3 (**10**) as a Ca^{2+}-mobilizing second messenger in 1983 (Streb *et al.*, 1983), several important physiological functions have been described for various inositol phosphates in eukaryotic cells (Duong *et al.*, 2018). Besides $InsP_6$, Ins(1,2,3,4,6)P_5 (**21**) and DL-Ins(1,2,3,4,5)P_5 (**22**) also showed inhibitory effects on the formation of hydroxyl radicals, and the 1,2,3 (equatorial-axial-equatorial) phosphate grouping was considered to be the corresponding iron binding site for this effect (Hawkins *et al.*, 1993). In addition, lower inositol phosphates might also contribute to the antioxidant defense *in vivo* by preventing the formation of reactive oxygen species (ROS) (Burgess and Gao, 2002). Ins(1,3,4,5)P_4 (**17**) was reported to have important roles in T cell, B cell and neutrophil cell development and function (Sauer and Cooke, 2010) as well as in NK cell maturation and responsiveness (Sauer *et al.*, 2013). Ins(3,4,5,6)P_4 (**20**) blocks Cl^- conductance through the chloride channel ClC-3 *in vivo* (Mitchell *et al.*, 2008). Duo to the many functions of ClC-3, Ins(3,4,5,6)P_4 (**20**) has been discussed as a potential regulator of neuronal development, tumor cell migration, bone modelling, apoptosis and inflammatory responses (Mitchell *et al.*, 2008). In addition, there are studies indicating that Ins(1,3,4,5,6)P_5 (**24**) is a specific PtdIns(3,4,5)P_3 competitor and able to block the serine phosphorylation and the kinase activity of the phosphoinositide 3-kinase/serine/threonine protein kinase B (PI3K/Akt) signaling pathway leading to antiangiogenic, proapoptotic and anticancer activities *in vitro* and *in vivo* (Maffucci *et*

al., 2005; Piccolo *et al.*, 2004). Furthermore, Ins(1,3,4,5,6)P_5 (**24**) was reported to sensitize breast, ovarian, and lung cancer cell lines to chemotherapeutic drugs (Piccolo *et al.*, 2004). In this regard, Ins(1,3,4,5,6)P_5 (**24**) and Ins(1,4,5,6)P_4 (**20**) were reported to have an even higher anticancer activity than InsP_6 (Ferry *et al.*, 2002).

Despite the described physiological functions, the contribution of dietary inositol phosphates to the pools found in the human body is discussed controversially, and no inositol phosphate carrier has been identified in the gut so far (Duong *et al.*, 2018). However, pinocytosis (Ferry *et al.*, 2002) and receptor-mediated endocytosis (Vucenik and Shamsuddin, 1994) have been discussed as possible mechanisms for the inositol phosphate uptake. Wilson *et al.* (2015) reasoned that an intestinal phytic acid absorption is unlikely because they did not detect any phytic acid in human plasma or urine via a specific titanium dioxide purification method. In contrast, the positive health effects associated with phytic acid, such as its anticancer activity, suggest the intestinal absorption of either phytic acid or its degradation products (Schlemmer *et al.*, 2009). In addition, the results of both animal and human studies indicate that the inositol phosphate levels of various tissues and biological fluids just as the urinary excretion of these compounds are related to the oral intake of phytic acid (Grases *et al.*, 2001b; Grases *et al.*, 2001a; Sakamoto *et al.*, 1993).

Taking the potential physiological properties of lower inositol phosphates into consideration, the *IPK1 lpa* soybean mutant ZC-*lpa* and its homozygous *lpa* mutant progenies with their remarkably high contents of lower inositol phosphates might be a valuable source of these bioactive compounds. However, the knowledge gaps regarding both the bioavailability and dose-response relationships of inositol phosphates emphasize the necessity for further investigations to draw valid conclusions regarding the nutritional evaluation of inositol phosphates for both human and animal nutrition.

Metabolite Profiles

Despite their outstanding nutritional value, soybean seeds also contain other natural antinutrients besides phytic acid such as heat-stable raffinose oligossacharides (RFOs) (Liener, 1981). RFOs are sucrose-based oligosaccharides and consist of linear chains of galactosyl residues which are attached to the glucose moiety of sucrose via an α-(1→6) glycosidic linkage (Avigad and Dey, 1997). The two RFOs raffinose and

stachyose are among the dominating soluble carbohydrates in soybean seeds besides sucrose (Hsu *et al.*, 1973) and act as storage carbohydrates, which can be used during early stage of germination (Peterbauer and Richter, 2001). Humans and nonruminant animals cannot hydrolyze the *α*-galactosidic linkage of RFOs because they lack *α*-galactosidase (EC 3.2.1.22) in their distal digestive tract (Peterbauer and Richter, 2001). Thus, RFOs pass into the colon, where they are fermented by intestinal bacteria, resulting in the production of gastrointestinal gas. The presence of RFOs in soybean seeds therefore causes flatulence, diarrhea and other digestive distress (Price *et al.*, 1988).

The *MIPS1* mutation of the primary *lpa* soybean mutant TW-*lpa* was shown to result in significantly reduced concentrations of the undesirable oligosaccharides raffinose and stachyose; an unintended but positive side effect of the reduced $InsP_6$ contents (Frank *et al.*, 2009). In this study, TW-1-M-*lpa*, a natural mutant of TW-*lpa* possessing the same 2 bp deletion in the third exon of *MIPS1* (Yuan *et al.*, 2017), was used for metabolite profiling. TW-1-M-*lpa* was shown to possess the same *MIPS1*-induced metabolic phenotype as TW-*lpa* with reduced contents of RFOs and galactosyl cyclitols. Even after cross-breeding with the commercial cultivar ZXD, this mutation-induced metabolic signature was maintained and consistently expressed over generations and different growing seasons in homozygous *lpa* mutant progenies.

In addition to the low RFO contents, the *MIPS1* mutants and their homozygous *lpa* mutant progenies showed increased concentrations of sucrose compared to their respective wild-types. High sucrose contents are a desirable trait in soybean seeds, as sucrose is the major source of energy for fermentation and contributes to the taste and flavor of soyfoods such as tofu and soymilk (Hou *et al.*, 2009). In addition, the combination of high sucrose and low RFO contents increases the metabolizable energy, making such mutants especially attractive for the feed industry (Hagely *et al.*, 2013).

The obtained results are encouraging for breeders because they demonstrated the stability of the desirable *MIPS1*-specific metabolic phenotype upon cross-breeding, i.e. reductions in the contents of $InsP_6$ and RFOs in combination with increased contents of sucrose. Taking into consideration the nutritional as well as environmental advantages of *lpa* crops, and the fact that high sucrose and low RFO contents are one of the most valuable traits for soybean breeding in food and feed industries (Hou *et al.*,

2009), the *MIPS1* mutants and their homozygous *lpa* progenies seem to be valuable germplasms for future soybean breeding programs.

4.4 Outlook

The elaborated HPIC data demonstrated that the homozygous *lpa* double mutant soybean seeds simultaneously carrying the *MIPS1* and *IPK1* mutation exhibited the lowest phytic acid contents and only moderate accumulations of lower inositol phosphate isomers. From a nutritional point of view, such an *lpa* trait is especially desirable and promising. The *IPK1* mutation has been shown to only cause minor changes in the contents of low molecular weight constituents, i.e. increased concentrations of *myo*-inositol, phosphoric acid and syringic acid in the *lpa* mutant compared to its respective wild-type (Frank *et al.*, 2009). In contrast, the *MIPS1* mutation resulted in nutritionally highly attractive reductions of raffinose oligosaccharides and galactosyl cyclitols as well as increased concentrations of sucrose besides the intended effect of phytic acid reduction (Frank *et al.*, 2009; Goßner *et al.*, 2019). Future investigations of the metabolite profiles of *lpa* double mutants need to demonstrate whether these desirable mutation-induced metabolic changes which have been observed in single *lpa* mutants are also consistently expressed in double *lpa* mutants.

Cross-breeding is not only applied to generate *lpa* cultivars with improved nutritional properties but also to improve their agronomic performance (Spear and Fehr, 2007; Zhao *et al.*, 2008). In the present study, only limited amounts of sample material were available which did not allow the simultaneous investigation of the agronomic performance of the homozygous *lpa* mutant soybean progenies resulting from the cross-breeding experiments. However, such agronomic data comprising germination rate, field emergence, yield, and the responses to various biotic and abiotic stresses are indispensable in subsequent studies to evaluate the agronomic competitiveness of *lpa* mutant progenies and to draw final conclusions regarding their applicability for commercialization.

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6 APPENDIX

6.1 Supporting Information Publication I

Goßner, S.; Yuan, F.; Zhou, C.; Tan, Y.; Shu, Q.; Engel, K. H.

Impact of Cross-Breeding of *Low Phytic Acid MIPS1* and *IPK1* Soybean (*Glycine max* L. Merr.) Mutants on their Contents of Inositol Phosphate Isomers.

J. Agric. Food Chem. 2019, *67*(1), 247-257.

https://pubs.acs.org/doi/full/10.1021/acs.jafc.8b06117;

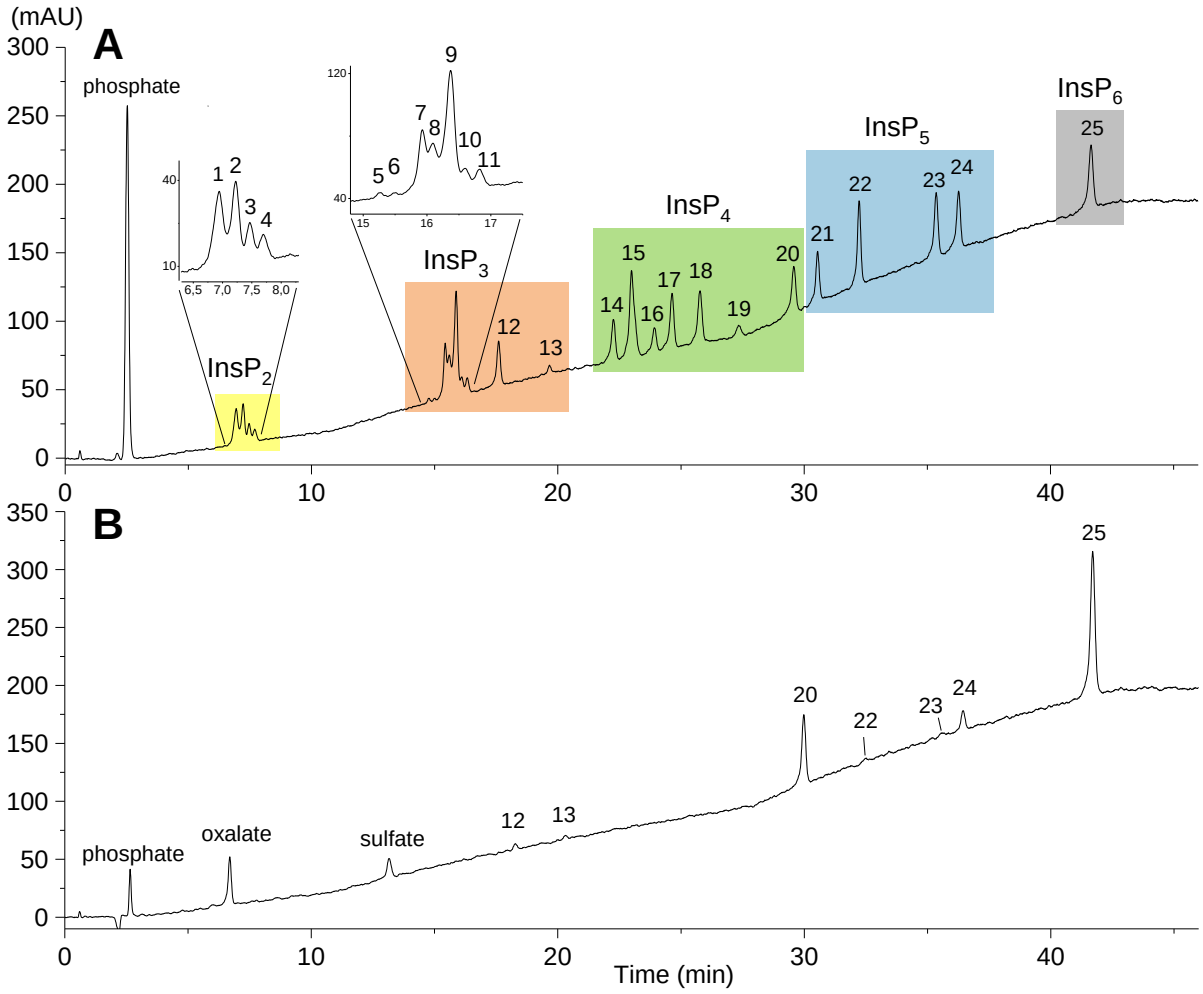

Figure S1. HPIC-chromatograms of the reference standard solution InsP-Mix (5% diluted in deionized water) containing 32 inositol phosphate isomers which were separated into 25 peaks (A), and of the *IPK1* mutant ZC-*lpa* (B). Peak numbers correspond to those given in Table S2.

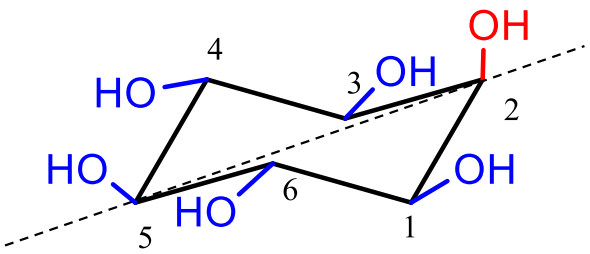

Figure S2. *Myo*-inositol (Ins) in the stable chair conformation with one axial (C2 in red) and five equatorial (blue) hydroxyl groups. The molecule can be divided into two non-superimposable mirror image halves between C2 and C5, illustrated by the dashed line. Phosphorylation at positions C2 or C5 maintains the plane of symmetry and therefore results in achiral molecules. Phosphorylation at C1, C3, C4 or C6 eliminates the plane of symmetry leading to chiral inositol phosphates (adapted from Murthy, 2006).

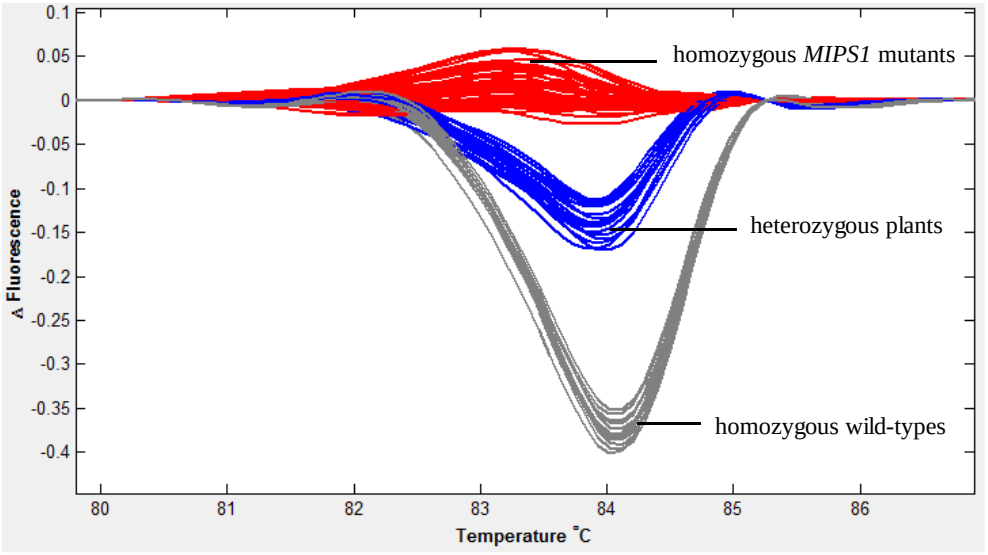

Figure S3. Exemplary genotyping results for the *MIPS1* mutation obtained by CADMA-HRM analysis of F_5 progenies from the cross TW-1-M-*lpa* x ZXD and the corresponding crossing parents TW-1-M-*lpa* (*MIPS1* mutant) and ZXD (WT).

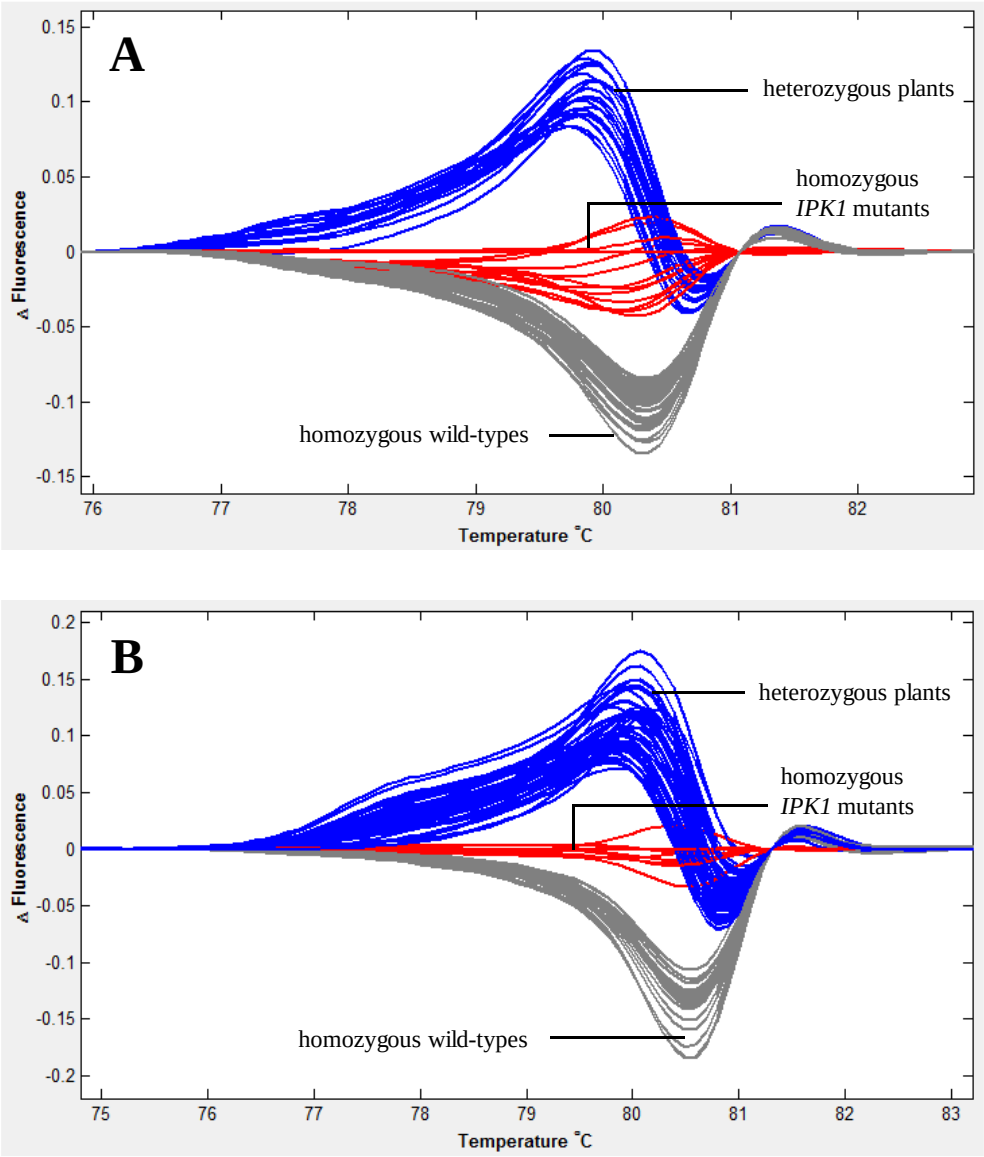

Figure S4. Exemplary genotyping results for the *IPK1* mutation obtained by CADMA-HRM analysis of F_5 progenies of the crosses ZC-*lpa* x Cu (A) and ZC-*lpa* x ZC-3 (B).

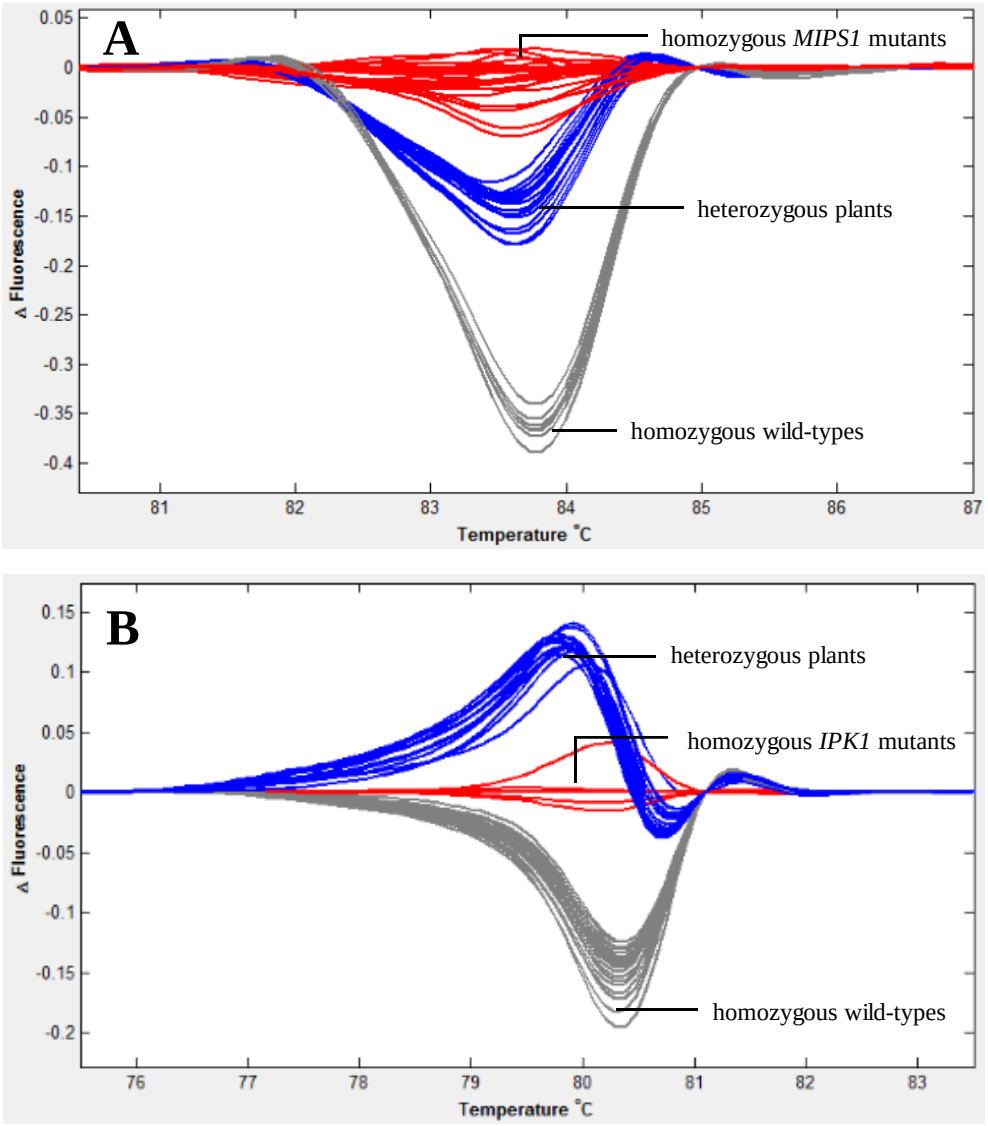

Figure S5. Exemplary genotyping results for the double mutants obtained by CADMA-HRM analysis of F_5 progenies of the cross ZC-*lpa* x TW-1-M-*lpa*: test for *MIPS1* (A), test for *IPK1* (B).

Table S1. Soybean sample material.

A Wild-type soybean lines

material	location	season
Taiwan 75	Hangzhou, China	spring 2015
Zhechun No. 3 (ZC-3)	Hangzhou, China	spring 2014
Zhexiandou No. 4 (ZXD)	Hangzhou, China	spring 2014
Cu	Hangzhou, China	spring 2014

B Lpa mutant soybean lines

lpa mutant	wild-type progenitor	affected gene	location	season
Gm-lpa-TW-1 (TW-*lpa*)	Taiwan 75	*GmMIPS1*	Hangzhou, China Hangzhou, China	spring 2014 spring 2015
Gm-lpa-TW-1-M (TW-1-M-*lpa*)	Taiwan 75	*GmMIPS1*	Hangzhou, China Haining, China	spring 2014 autumn 2016
Gm-lpa-ZC-*2* (ZC-*lpa*)	ZC-3	*GmIPK1*	Hangzhou, China Haining, China	spring 2014 autumn 2016

C Progenies of soybean crosses (x, available; n.a., not available)

crosses	generation	location	season	progenies			
				homozygous *MIPS1* mutants	homozygous *IPK1* mutants	homozygous wild-types	double mutants (*MIPS1 & IPK1*)
TW-1-M-*lpa* x	F_3	Hangzhou, China	spring 2014	x	n.a	x	n.a
ZXD	F_5	Hangzhou, China	spring 2015	x	n.a.	x	n.a
ZC-*lpa* x Cu	F_5	Hangzhou, China	spring 2014	n.a.	x	x	n.a
ZC-*lpa* x	F_5	Hangzhou, China	spring 2014	n.a.	x	x	n.a
ZC-3	F_6	Hangzhou, China	autumn 2014	n.a.	x	x	n.a
	F_3	Hangzhou, China	spring 2014	x	n.a	x	x
ZC-*lpa* x	F_5	Hangzhou, China	spring 2015	n.a.	n.a	x	x
TW-1-M-*lpa*	F_8	Haining, China	autumn 2016	n.a.	n.a	x	x

Table S2. Inositol phosphates of an in-house reference standard solution separated by HPIC. Both enantiomers are indicated in the D-configuration, if applicable.

	inositol phosphate isomer (no.)	corresponding enantiomer (no.)	retention time (min)
InsP$_2$	Ins(1,3)P$_2$ (**1**) [b]	–	6.9
	Ins(1,2)P$_2$ (**2**) [b]	Ins(2,3)P$_2$ (**2**)	7.2
	Ins(2,4)P$_2$ (**3**) [b]	Ins(2,6)P$_2$ (**3**)	7.5
	Ins(1,4)P$_2$ (**3**) [b]	Ins(3,6)P$_2$ (**3**)	7.5
	Ins(4,5)P$_2$ (**4**) [a]	Ins(5,6)P$_2$ (**4**)	7.7
	Ins(2,5)P$_2$ (**4**) [b]	–	7.7
InsP$_3$	Ins(1,3,5)P$_3$ (**5**) [b]	–	14.8
	Ins(2,4,6)P$_3$ (**6**) [b]	–	15.0
	Ins(1,3,4)P$_3$ (**7**) [b]	Ins(1,3,6)P$_3$ (**7**)	15.4
	Ins(1,2,4)P$_3$ (**8**) [b]	Ins(2,3,6)P$_3$ (**8**)	15.6
	Ins(2,3,5)P$_3$ (**8**) [b]	Ins(1,2,5)P$_3$ (**8**)	15.6
	Ins(1,2,3)P$_3$ (**9**) [b]	–	15.9
	Ins(1,2,6)P$_3$ (**9**) [b,]	Ins(2,3,4)P$_3$ (**9**)	15.9
	Ins(1,4,6)P$_3$ (**9**) [b]	Ins(3,4,6)P$_3$ (**9**)	15.9
	Ins(1,4,5)P$_3$ (**10**) [b]	Ins(3,5,6)P$_3$ (**10**)	16.1
	Ins(2,4,5)P$_3$ (**11**) [a]	Ins(2,5,6)P$_3$ (**11**)	16.3
	Ins(1,5,6)P$_3$ (**12**) [a]	Ins(3,4,5)P$_3$ (**12**)	17.6
	Ins(4,5,6)P$_3$ (**13**) [b]	–	19.7
InsP$_4$	Ins(1,2,4,6)P$_4$ (**14**) [b]	Ins(2,3,4,6)P$_4$ (**14**)	22.3
	Ins(1,2,3,5)P$_4$ (**14**) [b]	–	22.3
	Ins(1,2,3,4)P$_4$ (**15**) [b]	Ins(1,2,3,6)P$_4$ (**15**)	23.0
	Ins(1,3,4,6)P$_4$ (**15**) [b]	–	23.0
	Ins(1,2,4,5)P$_4$ (**16**) [b]	Ins(2,3,5,6)P$_4$ (**16**)	23.9
	Ins(1,3,4,5)P$_4$ (**17**) [b]	Ins(1,3,5,6)P$_4$ (**17**)	24.6
	Ins(1,2,5,6)P$_4$ (**18**) [b]	Ins(2,3,4,5)P$_4$ (**18**)	25.8
	Ins(2,4,5,6)P$_4$ (**19**) [b]	–	27.3
	Ins(1,4,5,6)P$_4$ (**20**) [a]	Ins(3,4,5,6)P$_4$ (**20**)	29.6
InsP$_5$	Ins(1,2,3,4,6)P$_5$ (**21**) [b]	–	30.6
	Ins(1,2,3,4,5)P$_5$ (**22**) [b]	Ins(1,2,3,5,6)P$_5$ (**22**)	32.2
	Ins(1,2,4,5,6)P$_5$ (**23**) [a]	Ins(2,3,4,5,6)P$_5$ (**23**) [b]	35.4
	Ins(1,3,4,5,6)P$_5$ (**24**) [a]	–	36.3
InsP$_6$	Ins(1,2,3,4,5,6)P$_6$ (**25**) [a]	–	41.7

[a] Identified by comparison of HPIC retention times with those of commercially obtained standards.

[b] Tentatively identified by assignment of the respective peak from the in-house reference standard solution on the basis of available HPIC data (Oates *et* al. 2014; Chen & Li, 2003).

Table S3. Overview of theoretically possible *myo*-inositol phosphate isomers (shown in D-configuration). Compounds with asterisks indicate substances which were detectable with the applied HPIC method.

InsP-compound	corresponding enantiomer
Ins(1)P_1	Ins(3)P_1
Ins(2)P_1	
Ins(4)P_1	Ins(6)P_1
Ins(5)P_1	
Ins(1,2)P_2*	Ins(2,3)P_2*
Ins(1,3)P_2*	
Ins(1,4)P_2*	Ins(3,6)P_2*
Ins(1,5)P_2	Ins(3,5)P_2
Ins(1,6)P_2	Ins(3,4)P_2
Ins(4,5)P_2*	Ins(5,6)P_2*
Ins(2,5)P_2*	
Ins(2,4)P_2*	Ins(2,6)P_2*
Ins(4,6)P_2	

InsP-compound	corresponding enantiomer
Ins(1,2,3)P_3*	
Ins(1,2,4)P_3*	Ins(2,3,6)P_3*
Ins(2,3,5)P_3*	Ins(1,2,5)P_3*
Ins(1,2,6)P_3*	Ins(2,3,4)P_3*
Ins(2,4,6)P_3*	
Ins(2,4,5)P_3*	Ins(2,5,6)P_3*
Ins(1,3,4)P_3*	Ins(1,3,6)P_3*
Ins(1,5,6)P_3*	Ins(3,4,5)P_3*
Ins(4,5,6)P_3*	
Ins(1,3,5)P_3*	
Ins(1,4,6)P_3*	Ins(3,4,6)P_3*
Ins(1,4,5)P_3*	Ins(3,5,6)P_3*
Ins(1,2,3,4)P_4*	Ins(1,2,3,6)P_4*

InsP-compound	corresponding enantiomer
Ins(1,3,4,5)P_4*	Ins(1,3,5,6)P_4*
Ins(1,2,5,6)P_4*	Ins(2,3,4,5)P_4*
Ins(2,4,5,6)P_4*	
Ins(1,4,5,6)P_4*	Ins(3,4,5,6)P_4*
Ins(1,2,4,5)P_4*	Ins(2,3,5,6)P_4*
Ins(1,2,4,6)P_4*	Ins(2,3,4,6)P_4*
Ins(1,3,4,6)P_4*	
Ins(1,2,3,5)P_4*	
Ins(1,2,3,4,5)P_5*	Ins(1,2,3,5,6)P_5*
Ins(1,2,4,5,6)P_5*	Ins(2,3,4,5,6)P_5*
Ins(1,3,4,5,6)P_5*	
Ins(1,2,3,4,6)P_5*	
Ins(1,2,3,4,5,6)P_6*	

Table S4. Limits of detection (LOD), limits of quantification (LOQ) and characteristics of calibration curves of different inositol phosphate isomers.

InsP isomer (no.)	LOD [µg/mL][1]	LOQ [µg/mL][1]	calibration range [µg/mL]	calibration levels	linearity (R^2)[2]	slope	intercept
Ins(4,5)P_2 (**4**)	1.1	3.4	0.7 – 22	6	> 0.9986	0.4152	-0.0471
Ins(2,4,5)P_3 (**11**)	1.2	3.6	1.4 – 43	6	> 0.9959	0.3963	-0.4314
Ins(1,4,5,6)P_4 (**20**)	0.5	1.4	0.7 – 170	9	> 0.9981	1.0104	0.9466
Ins(1,3,4,5,6)P_5 (**24**)	0.8	2.4	1.3 – 168	8	> 0.9963	0.5900	-0.7292
Ins(1,2,3,4,5,6)P_6 (**25**)	0.7	2.1	1.6 – 500	11	> 0.9998	0.6898	-1.6673

[1] LODs and LOQs expressed as µg/mL of injection volume (HPIC); determined on the basis of 100 µL injection volume. LODs and LOQs were calculated as 3.3 and 10 times the standard deviation of the response divided through the slope of the calibration curve, respectively (ICH, 2005).

[2] Each level injected in duplicate.

Table S5. Repeatabilities, reproducibilities and recovery rates of inositol phosphate isomers. All values were obtained by HPIC analyses of 50 mg and 400 mg commercially available soybean flour spiked with 880 µL of undiluted InsP-Mix, respectively.

		method applied for *IPK1* mutants (50 mg)			method applied for *MIPS1* mutants and WTs (400 mg)		
peak no.	inositol phosphate isomer	repeatability[1] RSD[4] [%]	reproducibility[2] RSD[4] [%]	recovery rate[3] [%]	repeatability[1] RSD[4] [%]	reproducibility[2] RSD[4] [%]	recovery rate[3] [%]
1	Ins(1,3)P_2	2.3	4.5	102	0.5	1.3	95
2	Ins(1,2)P_2	0.5	0.5	105	0.5	1.4	90
3	Ins(2,4)P_2, Ins(1,4)P_2	2.7	1.4	109	1.4	3.5	87
4	Ins(4,5)P_2, Ins(2,5)P_2	2.0	2.7	96	3.5	2.9	102
5	Ins(1,3,5)P_3	1.2	8.0	96	2.2	7.2	88
6	Ins(2,4,6)P_3	4.4	8.8	90	1.2	13.3	112
7	Ins(1,3,4)P_3	1.9	0.7	100	2.1	1.7	100
8	Ins(1,2,4)P_3, Ins(2,3,5)P_3	3.2	1.0	100	0.7	3.5	97
9	Ins(1,2,3)P_3, Ins(1,2,6)P_3, Ins(1,4,6)P_3	3.0	1.7	107	1.1	1.7	98
10	Ins(1,4,5)P_3	4.7	4.4	104	0.2	2.8	92
11	Ins(2,4,5)P_3	5.9	7.5	100	0.9	4.4	88
12	Ins(1,5,6)P_3	0.7	5.0	100	0.4	1.1	92
13	Ins(4,5,6)P_3	3.7	10.3	101	0.8	1.8	88
14	Ins(1,2,4,6)P_4, Ins(1,2,3,5)P_4	2.5	6.2	102	1.6	2.7	87
15	Ins(1,2,3,4)P_4, Ins(1,3,4,6)P_4	1.1	1.8	104	1.5	2.4	95
16	Ins(1,2,4,5)P_4	2.6	4.1	102	1.3	6.4	94
17	Ins(1,3,4,5)P_4	2.9	3.7	99	0.7	1.7	100
18	Ins(1,2,5,6)P_4	1.6	2.4	102	0.6	1.0	100
19	Ins(2,4,5,6)P_4	1.6	2.1	102	1.0	1.3	90
20	Ins(1,4,5,6)P_4	1.2	1.3	101	1.0	3.1	101
21	Ins(1,2,3,4,6)P_5	1.9	3.2	100	0.4	2.4	100
22	Ins(1,2,3,4,5)P_5	0.9	4.3	104	1.1	3.7	102
23	Ins(1,2,4,5,6)P_5	6.9	7.1	103	1.2	2.8	103
24	Ins(1,3,4,5,6)P_5	3.9	7.8	104	1.0	2.6	98

Table S5. (continued)

25	Ins(1,2,3,4,5,6)P_6	1.1	1.8	113	0.6	0.7	92
	mean ± SD	2.6 ± 1.6	4.1 ± 2.8	102 ± 4	1.1 ± 0.7	3.1 ± 2.6	96 ± 6
	min	0.5	0.5	90	0.2	0.7	87
	max	6.9	10.3	113	3.5	13.3	112

[1] single sample work-up, n = 3 injections

[2] three repeated sample work-ups obtained by the same operator on three consecutive days

[3] triplicate analysis of recovery rates of inositol phosphates; recovery rate [%] = [peakarea(spiked sample) – peakarea(unspiked sample)] / peakarea(amount spiked)

[4] relative standard deviation

Table S6. Sequence of *GmIPK1* and *GmMIPS1* genes in wild-type (WT) and *lpa* mutant soybeans and mutation-specific primers used for differentiating homozygous WTs and homozygous *lpa* mutants by CADMA-HRM (Yuan *et* al., 2007, Yuan *et* al., 2012; Tan *et* al., 2016).

	sequence of genes (5'-3')[1]		primers used for CADMA-HRM genotyping	expected product size (bp)
GmIPK1	WT	1514 GGAGAG**g**TACAT…	IPK1-OLP: 5' GCACCAAACTCTGAAATTGC 3'	191
			IPK1-F1: 5' CTCAGCTTCACCCCTTTC 3'	183
	lpa	1514 GGAGAG**a**TACAT…	IPK1-R: 3' CCGTAATTTAGAGACTCAATC 5'	183
GmMIPS1	WT	459 CA**ag**ATTCA…	MIPS1-OLP: 5' CTCAGGGGCATTTCATGGGC 3'	188
			MIPS1-F1: 5' TTTTGTTTTCGTTTTGCT 3'	180
	lpa	*459 CA**--**ATTCA…*	MIPS1-R: 3' ACTATGTACGGGTGAAC 5'	180

[1] Nucleotides in capital letters are shared between wild-type and *lpa* mutant alleles and those in bold lower case are different between wild-type and *lpa* mutant alleles.

6.2 Supporting Information Publication II

Goßner, S.; Yuan, F.; Zhou, C.; Tan, Y.; Shu, Q.; Engel, K. H.

Stability of the Metabolite Signature Resulting from the *MIPS1* Mutation in *Low Phytic Acid* Soybean (*Glycine max* L. Merr.) Mutants upon Cross-Breeding.

J. Agric. Food Chem. 2019, *67*(17), 5043-5052.

https://pubs.acs.org/doi/full/10.1021/acs.jafc.9b00817;

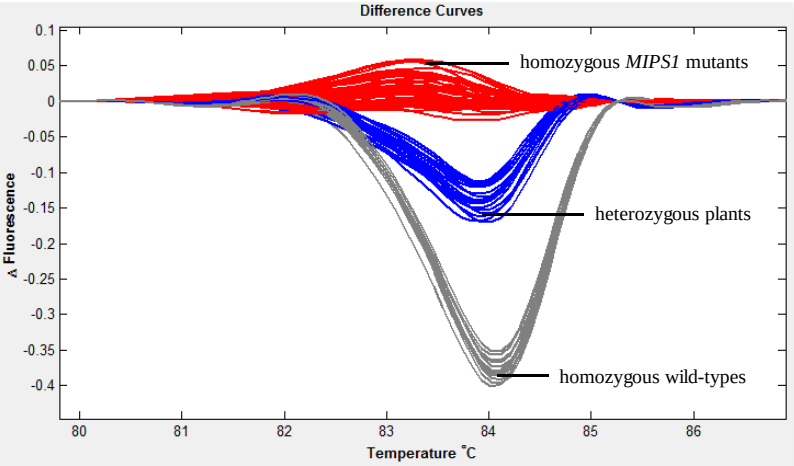

Figure S1. Genotyping results obtained by CADMA-HRM analysis of homozygous *MIPS1* mutants, homozygous wild-types (WT) and heterozygous F_3 and F_5 progenies of the cross TW-1-M-*lpa* x ZXD and their corresponding crossing parents.

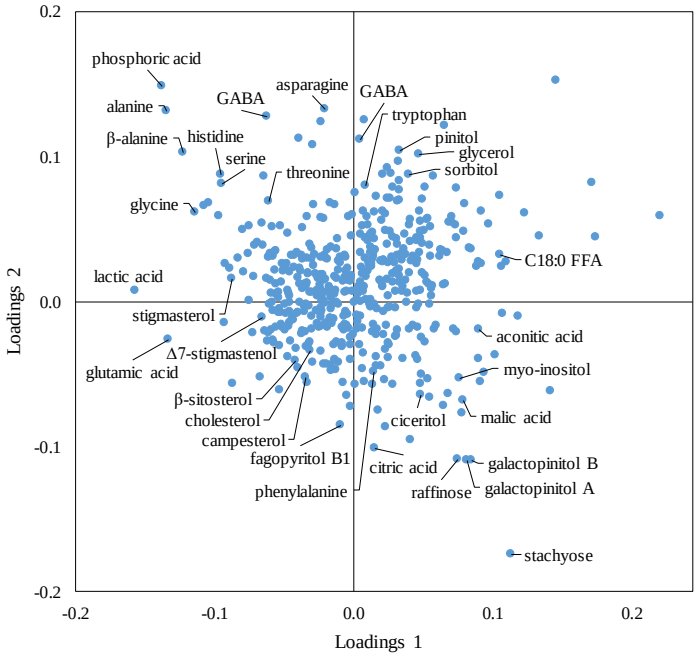

Figure S2. PCA loading plot of the combined metabolite profiling data of soybean seeds from the crossing parents TW-1-M-*lpa* and ZXD as well as the homozygous wild-type and homozygous *lpa* mutant F_3 and F_5 progenies.

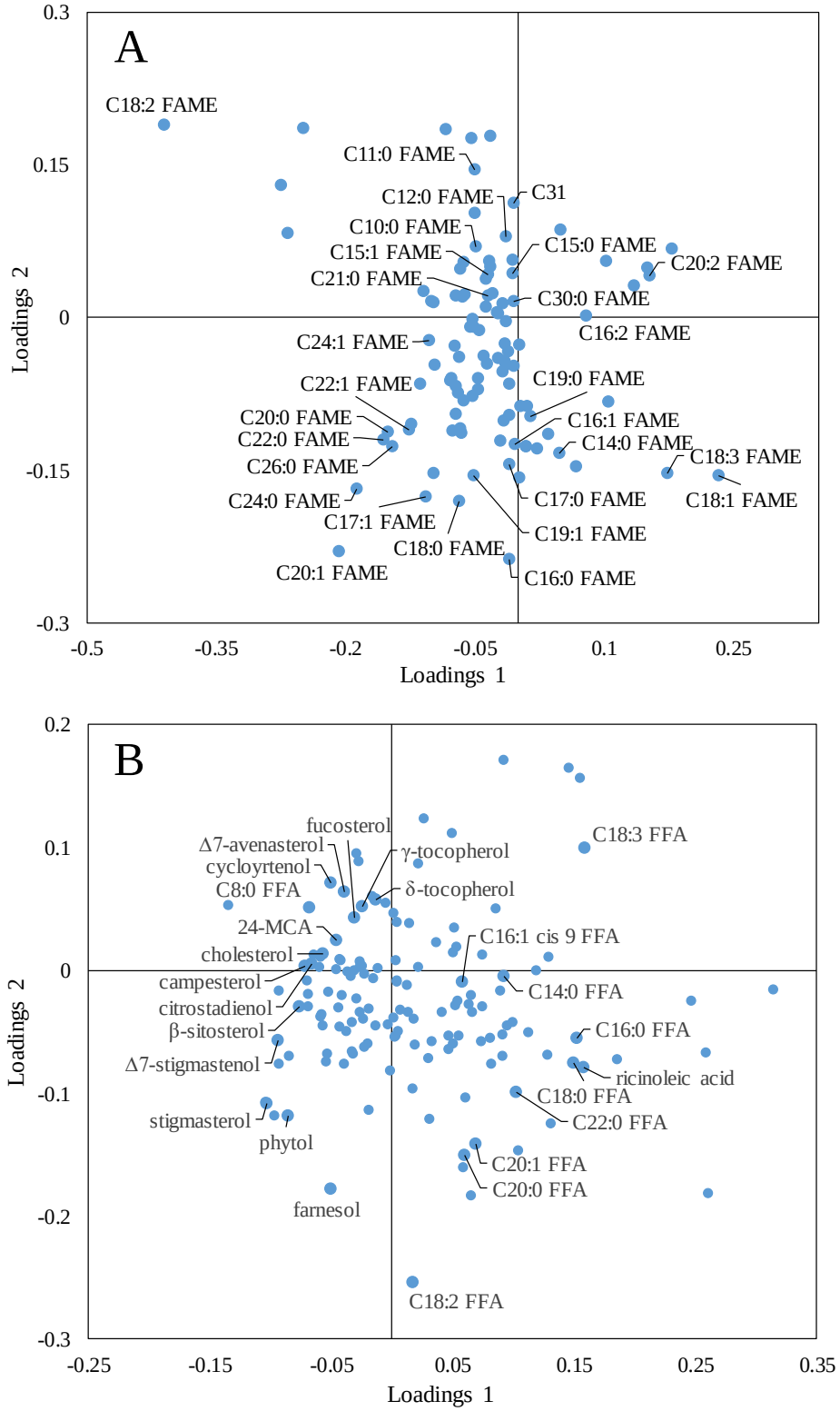

Figure S3. PCA loading plots from the lipophilic fractions I and II of the metabolite profiling data of soybean seeds from the crossing parents TW-1-M-*lpa* and ZXD as well as the homozygous wild-type and homozygous *lpa* mutant F_3 and F_5 progenies.

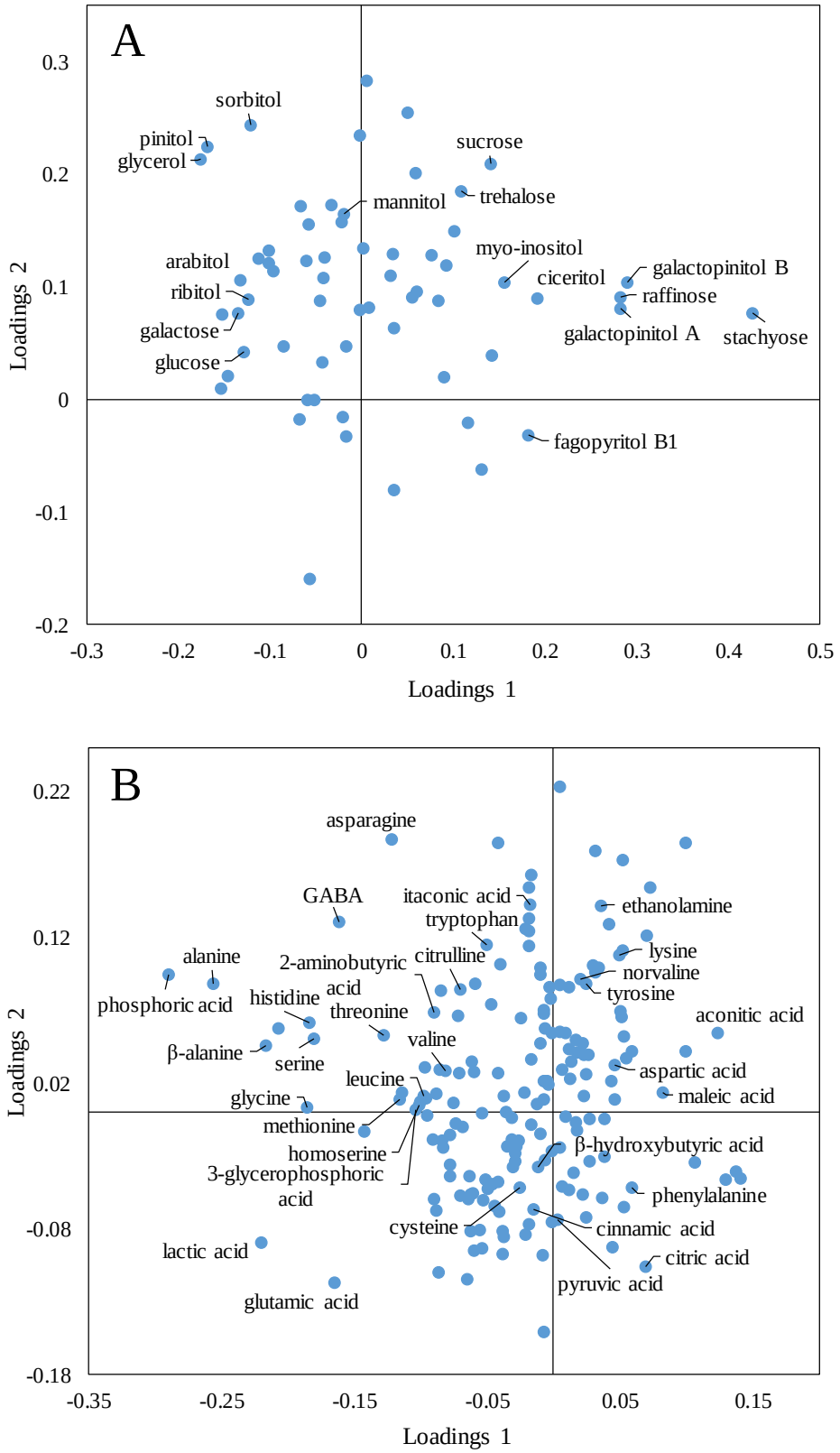

Figure S4. PCA loading plots from the polar fractions III and IV of the metabolite profiling data of soybean seeds from the crossing parents TW-1-M-*lpa* and ZXD as well as the homozygous wild-type and homozygous *lpa* mutant F_3 and F_5 progenies.

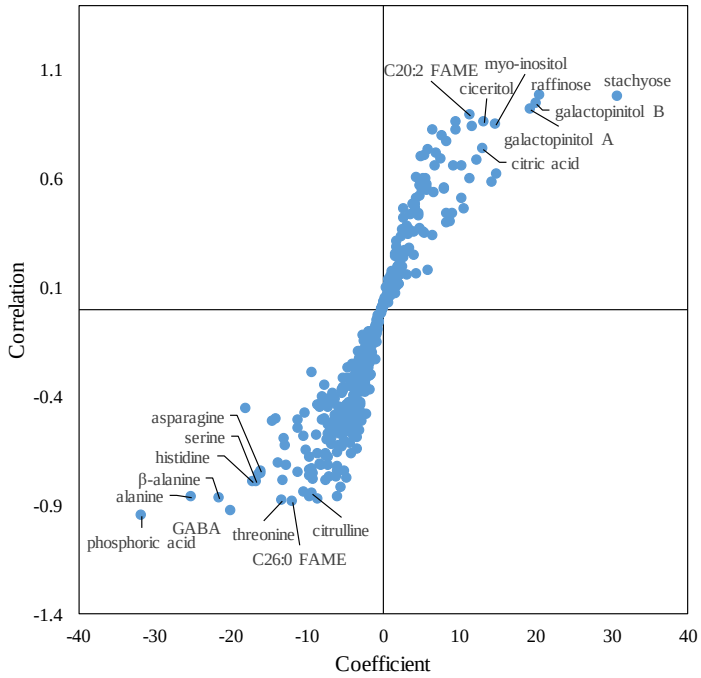

Figure S5. S-plot of the OPLS-DA model from the combined metabolite profiling data of homozygous wild-type and homozygous *lpa* mutant soybean seeds.

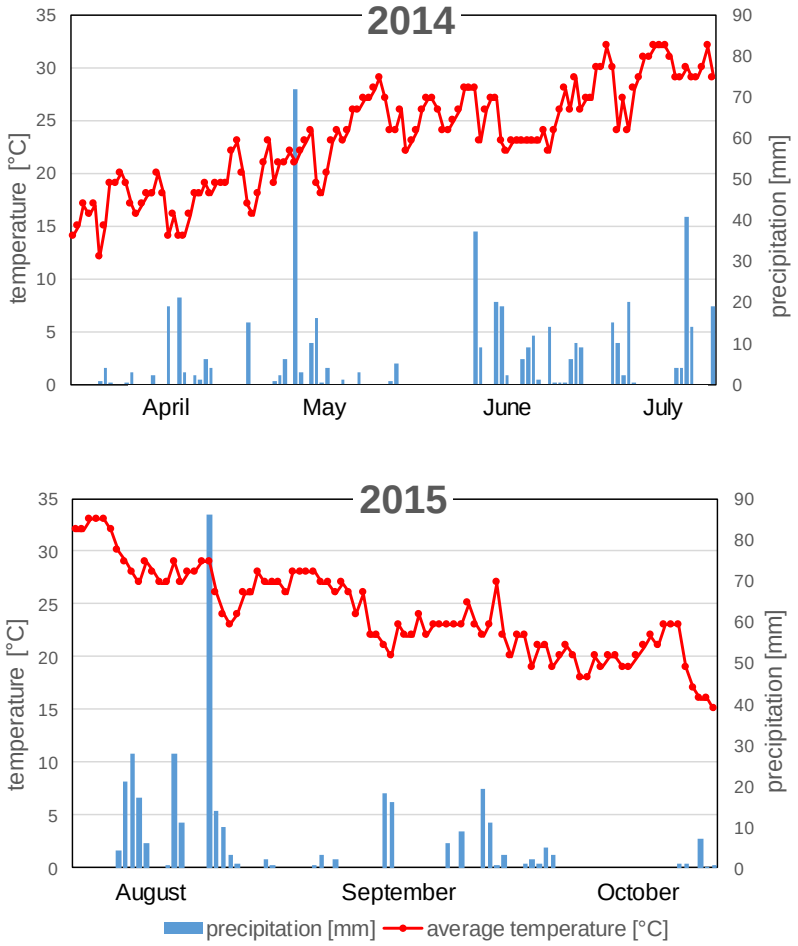

Figure S6. Average temperature and mean precipitation for the growing periods spring (April-July) 2014 and autumn (August-October) 2015 in Hangzhou, China (http://www.wunderground.com).

Table S1. Chromatographic and mass spectral data of compounds identified in fraction I.

no.	compound	ident.[a]	RRT[b]	M[c]	major fragment ions [(m/z), rel. intensity (%)]
saturated FAMEs[d]					
1	C10:0	A, B	0.268	186	74 (100), 87 (42), 55 (20), 143 (12), 59 (11)
2	C11:0	A, B	0.340	200	74 (100), 87 (46), 55 (21), 75 (12), 59 (10)
5	C12:0	A, B	0.428	214	74 (100), 87 (49), 55 (21), 75 (13), 57 (9)
6	C13:0	B	0.454	228	74 (100), 87 (54), 55 (24), 75 (16), 57 (12)
8	C14:0	A, B	0.574	242	74 (100), 87 (51), 55 (21), 75 (16), 143 (12)
11	C15:0	A, B	0.644	256	74 (100), 87 (55), 55 (24), 75 (21), 57 (13)
14	C16:0	A, B	0.716	270	74 (100), 87 (57), 55 (25), 75 (20), 57 (15)
17	C17:0	A, B	0.777	284	74 (100), 87 (58), 55 (27), 75 (23), 57 (17)
21	C18:0	A, B	0.845	298	74 (100), 87 (59), 75 (25), 55 (22), 57 (17)
24	C19:0	A, B	0.899	312	74 (100), 87 (85), 75 (28), 55 (26), 41 (25)
27	C20:0	A, B	0.955	326	74 (100), 87 (65), 75 (31), 55 (26), 57 (25)
28	C21:0	A, B	1.009	340	74 (100), 87 (66), 75 (33), 57 (24), 55 (18)
30	C22:0	A, B	1.062	354	74 (100), 87 (60), 75 (35), 57 (24), 55 (23)
32	C23:0	A, B	1.111	368	74 (100), 87 (73), 75 (42), 57 (34), 55 (22)
34	C24:0	A, B	1.160	382	74 (100), 87 (66), 75 (38), 57 (32), 55 (26)
36	C26:0	A, B	1.251	410	87 (100), 74 (82), 75 (64), 410 (23), 143 (23)
38	C28:0	A, B	1.338	438	87 (100), 74 (80), 75 (78), 438 (33), 83 (26)
39	C30:0	A, B	1.406	466	87 (100), 75 (86), 74 (73), 466 (33), 83 (28)
unsaturated FAMEs[d]					
7	C14:1	A, B	0.564	240	55 (100), 74 (53), 69 (50), 83 (31), 87 (28)
9	C15:1	B	0.625	254	55 (100), 74 (50), 69 (46), 83 (34), 87 (31)
13	C16:1	A, B	0.703	268	55 (100), 69 (66), 74 (61), 83 (48), 97 (39)
15	C17:1	A, B	0.760	282	55 (100), 69 (64), 74 (51), 83 (46), 96 (38)
20	C18:1	A, B	0.840	296	55 (100), 69 (66), 74 (56), 83 (53), 97 (44)
22	C19:1	B	0.884	310	55 (100), 69 (77), 83 (64), 97 (60), 67 (57)
26	C20:1	A, B	0.939	324	55 (100), 69 (70), 83 (50), 97 (45), 74 (45)

Table S1. (continued)

29	C22:1	A, B	1.047	352	55 (100), 69 (75), 83 (61), 97 (50), 74 (44)
33	C24:1	A, B	1.145	380	55 (100), 69 (81), 83 (64), 97 (55), 57 (43)
12	C16:2	B	0.688	266	67 (100), 81 (66), 95 (56), 82 (53), 96 (32)
19	C18:2	A, B	0.834	294	67 (100), 81 (84), 55 (57), 95 (54), 82 (46)
25	C20:2	A, B	0.935	322	67 (100), 81 (93), 82 (58), 95 (58), 55 (52)
18	C18:3	A, B	0.805	292	79 (100), 67 (89), 80 (73), 93 (54), 81 (49)
hydrocarbons					
3	C14	A, B	0.348	198	57 (100), 71 (87), 43 (73), 85 (51), 41 (38)
4	C15	A, B	0.421	212	57 (100), 71 (73), 43 (68), 85 (52), 41 (29)
10	C18	A, B	0.637	254	57 (100), 71 (87), 43 (62), 85 (56), 41 (31)
16	C20	A, B	0.768	282	57 (100), 71 (88), 85 (64), 43 (63), 41 (31)
23	C22	A, B	0.890	310	57 (100), 71 (87), 85 (57), 43 (54), 55 (21)
31	C26	A, B	1.102	366	71 (100), 57 (93), 85 (64), 43 (60), 55 (24)
35	C28	A, B	1.196	394	57 (100), 71 (99), 85 (74), 43 (60), 99 (24)
37	C31	A, B	1.327	436	57 (100), 71 (98), 85 (71), 43 (53), 99 (21)

[a] Identification according to (A) mass spectral data and retention times of reference compounds or (B) mass spectral data of NIST08 mass spectra library.
[b] Retention time relative to the internal standard tetracosane
[c] Molecular weight
[d] Fatty acid methyl esters

Table S2. Chromatographic and mass spectral data of compounds identified as trimethylsilyl derivatives in fraction II.

no.	compound	ident.[a]	RRT[b]	M[c]	major fragment ions [(m/z), rel. intensity (%)]
free fatty acids					
1	C8:0	B	0.182	216	75 (100), 117 (49), 201 (22), 129 (14), 74 (13)
2	C9:0	B	0.230	230	75 (100), 117 (63), 215 (21), 129 (19), 132 (17)
4	C12:0	A, B	0.386	272	75 (100), 117 (79), 257 (58), 129 (23), 132 (19)
6	C14:0	A, B	0.487	300	75 (100), 117 (87), 285 (69), 129 (31), 132 (21)
7	C15:0	A, B	0.535	314	117 (100), 75 (85), 299 (74), 129 (34), 74 (27)
9	C16:1 (*trans* 9)	B	0.568	326	75 (100), 79 (44), 117 (38), 311 (31), 129 (23)
10	C16:1 (*cis* 9)	A	0.575	326	75 (100), 117 (56), 311 (35), 129 (34), 81 (24)
11	C16:0	A, B	0.582	328	75 (100), 117 (93), 313 (69), 129 (36), 132 (27)
12	C17:0	A, B	0.625	342	75 (100), 117 (97), 327 (73), 129 (31), 132 (28)
15	C18:3	A	0.652	350	79 (100), 75 (97), 95 (45), 81 (41), 93 (41)
16	C18:2	A, B	0.654	352	75 (100), 81 (61), 79 (36), 95 (34), 337 (28)
17	C18:1 (*trans* 9)	B	0.659	354	75 (100), 117 (55), 129 (39), 339 (36), 145 (28)
18	C18:1 (*cis* 9)	A, B	0.663	354	75 (100), 117 (62), 339 (49), 129 (37), 81 (27)
19	C18:0	A, B	0.667	356	75 (100), 117 (97), 341 (77), 129 (38), 132 (30)
21	ricinoleic acid	A, B	0.691	442	187 (100), 73 (68), 75 (18), 188 (15), 103 (15)
22	C20:1	A, B	0.736	382	367 (100), 73 (96), 117 (79), 75 (77), 129 (75)
23	C20:0	B	0.746	384	117 (100), 75 (93), 369 (72), 129 (40), 34 (32)
25	C22:0	A, B	0.820	412	117 (100), 75 (88), 397 (62), 132 (35), 129 (32)
26	C23:0	A, B	0.854	426	117 (100), 75 (88), 411 (82), 129 (37), 132 (36)
27	C24:0	A, B	0.888	440	117 (100), 75 (80), 425 (59), 129 (39), 132 (38)
fatty alcohols					
3	C12:0	B	0.345	258	75 (100), 243 (66), 103 (30), 97 (25) 83 (24)
5	C14:0	A, B	0.448	286	75 (100), 271 (94), 103 (28), 89 (22), 97 (21)
8	C16:0	A, B	0.544	314	299 (100), 75 (69), 300 (24), 83 (22), 97 (17)
13	C18:0	A, B	0.633	342	327 (100), 75 (35), 328 (31), 103 (18), 97 (18)
24	C22:0	A, B	0.790	398	383 (100), 75 (55), 384 (31), 83 (18), 97 (16)

Table S2. (continued)

tocopherols					
28	δ-tocopherol	A, B	0.911	474	474 (100), 475 (42), 208 (36), 209 (31), 210 (16)
29	*γ*-tocopherol	A, B	0.944	488	488 (100), 490 (51), 223 (49), 222 (28), 224 (12)
30	*α*-tocopherol	A, B	0.988	502	502 (100), 237 (59), 503 (50), 236 (36), 238 (13)
sterols and triterpenic alcohols					
14	phytol	A, B	0.642	368	143 (100), 75 (49), 81 (21), 123 (17), 144 (15)
20	farnesol	B	0.673	294	81 (100), 93 (53), 107 (33), 95 (30), 121 (21)
31	cholesterol	A, B	0.996	458	129 (100), 329 (85), 75 (76), 368 (73), 81 (70)
32	campesterol	A, B	1.029	472	129 (100), 343 (75), 382 (69), 75 (63), 81 (57)
33	campestanol	A, B	1.032	474	207 (100), 215 (84), 75 (74), 73 (53), 216 (46)
34	stigmasterol	A, B	1.037	484	83 (100), 73 (69), 55 (67), 69 (57), 129 (45)
35	*ß*-sitosterol	A, B	1.055	486	129 (100), 95 (44), 81 (43), 357 (36), 396 (35)
36	Δ^5-avenasterol	B	1.059	484	386 (100), 296 (96), 281 (70), 387 (34), 257 (33)
37	fucosterol	B	1.066	484	129 (100), 81 (59), 95 (49), 105 (45), 119 (44)
38	Δ^7-stigmastenol	B	1.072	486	75 (100), 255 (71), 487 (63), 95 (48), 107 (46)
39	cycloartenol	A, B	1.074	498	95 (100), 81 (94), 109 (71), 408 (71), 107 (71)
40	Δ^7-avenasterol	B	1.075	484	343 (100), 75 (84), 386 (37), 253 (33), 81 (33)
41	24-methylene-cylcoartanol	A, B	1.090	512	95 (100), 81 (94), 107 (76), 93 (68), 75 (64)
42	citrostadienol	B	1.101	498	357 (100), 75 (92), 400 (47), 95 (40), 81 (39)

[a] Identification according to (A) mass spectral data and retention times of reference compounds or (B) mass spectral data of NIST08 mass spectra library.
[b] Retention time relative to the internal standard 5*α*-cholestan-3*β*-ol
[c] Molecular weight of trimethylsilyl derivative

Table S3. Chromatographic and mass spectral data of compounds identified as trimethylsilyl derivatives in fraction III.

no.	compound	ident.[a]	RRT[b]	M[c]	major fragment ions [(m/z), rel. intensity (%)]
sugar alcohols					
1	glycerol	A, B	0.272	308	73 (100), 205 (63), 147 (57), 103 (29), 117 (28)
2	xylitol	A, B	0.608	512	217 (100), 103 (59), 147 (45), 205 (37), 307 (34)
3	arabitol	B	0.613	512	217 (100), 307 (60), 117 (32), 319 (31), 79 (29)
4	ribitol	B	0.629	512	217 (100), 319 (65). 103 (62), 207 (42), 79 (38)
8	pinitol	A, B	0.705	554	147 (100), 260 (86), 133 (63), 318 (60), 217 (56)
12	mannitol	A, B	0.777	614	319 (100), 205 (51), 217 (35), 147 (32), 320 (29)
13	sorbitol	A, B	0.781	614	319 (100), 205 (57), 217 (54), 147 (45), 320 (33)
14	*chiro*-inositol	A, B	0.794	612	318 (100), 147 (84), 305 (77), 217 (44), 191 (34)
16	*myo*-inositol	A, B	0.880	612	305 (100), 217 (86), 191 (60), 318 (58), 147 (48)
24	ciceritol	C	1.647	1310	204 (100), 129 (29), 133 (26), 147 (24), 103 (23)
sugars					
5, 6, 7	fructose	A, B	0.684, 0.688, 0.696	900	204 (100), 217 (57), 147 (43), 437 (41), 205 (30)
9, 11	galactose	A, B	0.720, 0.751	540	204 (100), 191 (66), 217 (27), 147 (24), 205 (18)
10, 15	glucose	A, B	0.741, 0.806	540	204 (100), 191 (53), 147 (21), 205 (18), 217 (16)
17, 18	sucrose	A	1.182, 1.188	918	361 (100), 362 (39), 217 (27), 437 (22), 363 (19)
19	trehalose	A	1.244	918	361 (100), 191 (37), 362 (29), 217 (17), 363 (15)
20	galactopinitol A	C	1.251	932	204 (100), 129 (24), 217 (20), 103 (20), 205 (15)
21	galactopinitol B	C	1.288	932	204 (100), 103 (22), 205 (20), 129 (19), 147 (17)
22	fagopyritol B1	C	1.339	990	204 (100), 217 (25), 129 (24), 205 (20), 103 (15)
23	raffinose	A	1.522	1296	361 (100), 362 (32), 204 (30), 217 (27), 437 (23)
25	stachyose	A	2.038	1674	361 (100), 204 (77), 217 (46), 362 (33), 451 (24)

[a] Identification according to (A) mass spectral data and retention times of reference compounds, (B) mass spectral data of NIST08 mass spectra library or (C) data from a previous study (Frank *et* al. 2009).
[b] Retention time relative to the internal standard phenyl-*β*-D-glucopyranoside
[c] Molecular weight of trimethylsilyl derivative

Table S4. Chromatographic and mass spectral data of compounds identified as trimethylsilyl derivatives in fraction IV.

no.	compound	ident.[a]	RRT[b]	M[c]	major fragment ions [(m/z), rel. intensity (%)]
amino acids and amines					
4	alanine	A, B	0.261	233	116 (100), 147 (14), 117 (13), 118 (4), 190 (4)
5, 22	glycine	A, B	0.270, 0.451	291	174 (100), 147 (22), 86 (21), 175 (19), 248 (18)
6	hydroxylamine	B	0.281	249	73 (100), 133 (25), 146 (24), 119 (20), 86 (14)
9	2-aminobutyric acid	A, B	0.315	247	130 (100), 131 (12), 147 (11), 132 (4), 204 (3)
10	*ß*-alanine	A, B	0.326	305	102 (100), 147 (53), 176 (42), 218 (11), 75 (10)
12	valine	A, B	0.356	261	144 (100), 145 (13), 218 (12), 147 (10), 100 (6)
14	norvaline	A, B	0.377	261	144 (100), 145 (14), 147 (10), 146 (5), 75 (5)
16	leucine	A, B	0.410	275	158 (100), 159 (15), 102 (13), 147 (8), 160 (4)
17	ethanolamine	A, B	0.414	277	174 (100), 100 (28), 86 (27), 175 (21), 147 (18)
19, 35	GABA	A, B	0.431, 0.705	319	174 (100), 147 (31), 304 (24), 175 (18), 86 (14)
20	isoleucine	A, B	0.435	275	158 (100), 159 (13), 218 (12), 147 (9), 100 (7)
26	serine	A, B	0.507	321	204 (100), 218 (57), 100 (33), 147 (28), 205 (21)
28	threonine	A, B	0.542	335	219 (100), 117 (94), 218 (89), 101 (50), 291 (41)
29	*ß*-aminoisobutyric acid	A, B	0.584	319	102 (100), 147 (14), 176 (10), 103 (10), 218 (6)
30	homoserine	A, B	0.612	335	218 (100), 128 (50), 103 (32), 219 (22), 147 (14)
32	pyroglutamic acid	A, B	0.675	273	156 (100), 147 (15), 157 (14), 258 (8), 230 (7)
33	methionine	A, B	0.681	293	176 (100), 128 (97), 147 (19), 177 (18), 219 (12)
34	aspartic acid	A, B	0.689	349	232 (100), 100 (25), 147 (21), 233 (20), 218 (16)
36	cysteine	A, B	0.729	409	220 (100), 218 (82), 100 (31), 147 (21), 221 (19)
37	glutamic acid	A, B	0.804	363	246 (100), 128 (32), 247 (19), 156 (19), 147 (18)
38	phenylalanine	A, B	0.808	309	218 (100), 192 (80), 100 (32), 147 (28), 219 (21)
39	asparagine	A, B	0.857	348	116 (100), 132 (45), 231 (43), 75 (29), 188 (29)
40	*α*-aminoadipic acid	A, B	0.913	377	260 (100), 217 (61), 128 (35), 261 (26), 147 (22)
43	citrulline	A, B	1.033	391	70 (100), 73 (41), 100 (10), 142 (6), 171 (5)
47	histidine	A, B	1.122	443	154 (100), 254 (27), 155 (21), 255 (6), 156 (5)
49	lysine	A, B	1.150	434	174 (100), 156 (93), 317 (47), 128 (25), 230 (23)

Table S4. (continued)

50	tyrosine	A, B	1.154	397	73 (100), 218 (94), 219 (20), 100 (18), 147 (13)
52	tryptophan	A, B	1.419	420	202 (100), 73 (62), 203 (22), 291 (8), 204 (6)
organic and inorganic acids					
1	lactic acid	A, B	0.232	234	117 (100), 147 (93), 191 (22), 190 (18), 148 (16)
2	glycolic acid	A, B	0.238	220	147 (100), 177 (20), 148 (17), 149 (11), 205 (10)
3	threonic acid	A, B	0.249	424	73 (100), 147 (65), 292 (56), 220 (26), 205 (26)
7	pyruvic acid	A, B	0.289	248	147 (100), 73 (48), 217 (37), 148 (30), 149 (14)
8	*ß*-hydroxybutyric acid	A, B	0.301	248	147 (100), 117 (53), 191 (36), 148 (17), 75 (15)
11	malonic acid	B	0.332	248	147 (100), 75 (28), 148 (17), 149 (9), 74 (6)
13	*γ*-hydroxybutyric acid	A, B	0.365	248	117 (100), 143 (73), 75 (68), 147 (66), 233 (40)
15	phosphoric acid	A, B	0.404	314	299 (100), 300 (23), 314 (18), 301 (14) 133 (8)
18	maleic acid	A, B	0.427	260	147 (100), 73 (52), 148 (23), 245 (13), 75 (13)
21	succinic acid	A, B	0.439	262	147 (100), 73 (57), 148 (35), 247 (32), 75 (25)
23	glyceric acid	A, B	0.471	322	147 (100), 189 (56), 103 (36), 292 (35), 133 (32)
24	itaconic acid	A, B	0.471	274	147 (100), 73 (57), 148 (25), 215 (15), 75 (14)
25	fumaric acid	A, B	0.479	260	245 (100), 147 (46), 73 (39), 246 (28), 143 (18)
27	2-piperidinecarboxylic acid	B	0.511	273	156 (100), 157 (14), 147 (10), 158 (5), 230 (4)
31	malic acid	A, B	0.652	350	147 (100), 148 (21), 75 (13), 245 (12), 149 (11)
41	aconitic acid	A, B	0.942	390	147 (100), 229 (33), 75 (22), 148 (17), 317 (16)
42	3-glycerophosphoric acid	A, B	0.972	474	299 (100), 357 (83), 315 (24).129 (18), 358 (18)
44	citric acid	A, B	1.040	480	273 (100), 147 (87), 247 (29), 347 (26), 375 (24)
46	syringic acid	A, B	1.107	342	327 (100), 73 (73), 312 (70), 297 (68), 342 (63)
48	*p*-coumaric acid	A, B	1.139	308	219 (100), 293 (96), 249 (74), 308 (73), 75 (46)
51	mucic acid	B	1.290	642	147 (100), 75 (26), 261 (21), 158 (20), 148 (18)
others					
45	adenine	A, B	1.061	279	264 (100), 265 (23), 279 (23), 192 (9), 161 (8)

[a] Identification according to (A) mass spectral data and retention times of reference compounds or (B) mass spectral data of NIST08 mass.
spectra library

[b] Retention time relative to the respective internal standard *p*-chloro-L-phenylalanine

[c] Molecular weight of trimethylsilyl derivative

Table S5. Contents of phytic acid and relative peak responses of selected compounds (OPLS-DA VIP > 1.5) found to be statistically significantly different between the crossing parents ZXD and TW-1-M-*lpa* and between homozygous wild-type (HWT) und homozygous *lpa* mutant (HM) F_3 and F_5 progenies.[a,b]

	HWT parent	HM parent	F_3 progenies		F_5 progenies			
	ZXD	TW-1-M-*lpa*	HWT	HM	$F_{5\text{-}1}$ HWT	$F_{5\text{-}1}$ HM	$F_{5\text{-}2}$ HWT	$F_{5\text{-}2}$ HM
phytic acid	16.06 ± 0.07	9.90 ± 0.06	16.96 ± 0.27	8.61 ± 0.03	17.58 ± 0.07	10.64 ± 0.03	13.30 ± 0.09	9.42 ± 0.07
phosphoric acid	222 ± 7	711 ± 67	224 ± 33	1109 ± 253	133 ± 6	1539 ± 56	253 ± 11	1294 ± 57
sucrose	3366 ± 73	3058 ± 126	2360 ± 62	3057 ± 67	1802 ± 15	2792 ± 101	2408 ± 130	2904 ± 86
raffinose	314 ± 14	130 ± 10	398 ± 12	153 ± 4	350 ± 8	129 ± 2	323 ± 9	128 ± 1
stachyose	367 ± 27	7.9 ± 0.6	286 ± 24	11.0 ± 1.1	251 ± 1	14.0 ± 0.3	219 ± 17	12.6 ± 0.2
galactopinitol A	76.7 ± 1.4	3.5 ± 0.2	59.9 ± 1.1	6.0 ± 0.2	37.7 ± 1.5	7.2 ± 0.1	27.9 ± 0.5	8.2 ± 0.2
galactopinitol B	71.8 ± 1.4	4.3 ± 0.3	72.5 ± 1.1	8.9 ± 0.4	43.3 ± 0.8	7.3 ± 0.3	36.1 ± 2.2	7.7 ± 0.3
myo-inositol	17.4 ± 1.5	11.1 ± 0.9	28.1 ± 2.9	5.5 ± 0.7	32.1 ± 0.8	2.4 ± 0.1	12.3 ± 2.1	2.5 ± 0.1
ciceritol	5.7 ± 0.2	0.7 ± 0.1	13.1 ± 0.4	0.8 ± 0.1	5.3 ± 0.1	1.8 ± 1.6	4.1 ± 0.7	0.8 ± 0.3
glycine	14.9 ± 0.8	31.7 ± 4.5	23.5 ± 3.1	60.2 ± 10.6	23.1 ± 0.1	154 ± 1	16.0 ± 0.9	52.1 ± 0.7
alanine	91.3 ± 1.3	388 ± 55	135 ± 8.5	552 ± 30	147 ± 1	1047 ± 32	141 ± 4	360 ± 6
valine	17.3 ± 0.4	20.3 ± 3.7	20.9 ± 0.2	38.6 ± 4.3	20.7 ± 0.9	29.9 ± 2.3	20.2 ± 0.4	29.2 ± 2.7
leucine	12.8 ± 0.1	27.3 ± 2.8	21.9 ± 0.3	40.7 ± 5.7	22.9 ± 1.2	30.7 ± 2.5	26.1 ± 0.3	44.7 ± 3.9
serine	11.9 ± 0.1	28.3 ± 0.8	14.2 ± 2.8	51.0 ± 8.8	11.9 ± 0.1	132 ± 7	10.5 ± 0.2	34.4 ± 2.3
homoserine	0.6 ± 0.1	1.9 ± 0.6	2.3 ± 0.2	3.1 ± 0.9	1.7 ± 0.1	6.1 ± 0.3	1.6 ± 0.0	3.3 ± 0.1
threonine	10.2 ± 0.1	19.2 ± 0.8	9.3 ± 0.6	26.4 ± 4.5	7.9 ± 0.4	41.9 ± 5.9	9.3 ± 0.4	19.9 ± 2.5
methionine	3.7 ± 0.2	7.4 ± 0.7	6.6 ± 1.2	10.0 ± 1.6	6.2 ± 0.4	19.6 ± 0.4	5.2 ± 0.1	11.0 ± 0.2
phenylalanine	18.9 ± 0.7	18.6 ± 1.4	21.7 ± 0.5	21.0 ± 0.4	34.7 ± 1.2	13.0 ± 0.9	30.4 ± 3.3	18.4 ± 0.3
tryptophan	53.9 ± 1.3	89.2 ± 7.7	42.4 ± 3.7	109 ± 12	45.1 ± 0.6	53.6 ± 1.7	38.1 ± 0.5	69.1 ± 0.6
histidine	13.6 ± 0.5	38.7 ± 2.5	18.9 ± 2.7	72.0 ± 7.8	22.1 ± 0.8	155 ± 10	10.3 ± 1.0	42.0 ± 2.3
asparagine	2.7 ± 0.3	38.1 ± 4.1	14.9 ± 3.4	56.2 ± 10.4	2.3 ± 0.1	41.4 ± 10.4	1.9 ± 0.6	6.6 ± 0.6
β-alanine	16.7 ± 0.5	76.5 ± 11.4	15.3 ± 2.1	91.6 ± 12.2	34.6 ± 0.3	250 ± 1	10.7 ± 0.5	99.3 ± 1.9
citrulline	2.6 ± 0.5	6.3 ± 1.1	3.9 ± 0.7	6.3 ± 0.3	1.9 ± 0.1	6.8 ± 0.8	2.1 ± 0.2	4.1 ± 0.4
2-aminobutyric acid	0.6 ± 0.2	1.8 ± 0.4	1.4 ± 0.0	3.6 ± 0.4	0.6 ± 0.1	4.3 ± 0.2	0.6 ± 0.1	1.4 ± 0.2

Table S5. (continued)

GABA	16.7 ± 0.1	165 ± 6	25.2 ± 4.9	117 ± 15	25.3 ± 0.2	116 ± 1	16.0 ± 0.7	62.0 ± 1.5
citric acid	1118 ± 73	651 ± 22	1084 ± 74	885 ± 138	1013 ± 18	945 ± 66	1352 ± 68	839 ± 94
syringic acid	13.4 ± 0.5	13.7 ± 0.8	12.1 ± 3.5	17.0 ± 3.8	10.1 ± 0.4	16.6 ± 1.0	10.8 ± 0.3	17.8 ± 0.6
mucic acid	1.5 ± 0.1	3.6 ± 0.4	2.6 ± 0.2	5.4 ± 1.1	2.5 ± 0.3	7.1 ± 1.6	7.0 ± 1.2	6.5 ± 1.1
γ-hydroxybutyric acid	0.9 ± 0.2	6.5 ± 0.9	2.0 ± 0.1	4.2 ± 0.3	1.2 ± 0.2	2.2 ± 0.1	1.3 ± 0.0	2.0 ± 0.1
3-glycerophosphoric acid	3.7 ± 0.4	4.7 ± 0.2	3.2 ± 0.0	7.9 ± 0.5	2.7 ± 0.2	10.5 ± 0.9	3.9 ± 0.3	11.9 ± 0.8
C20:0 FAME	323 ± 15	521 ± 15	436 ± 8	408 ± 14	427 ± 7	502 ± 8	413 ± 10	491 ± 4
C24:0 FAME	183 ± 8	380 ± 5	253 ± 6	290 ± 17	292 ± 5	437 ± 12	277 ± 7	410 ± 7
C26:0 FAME	18.0 ± 0.9	43.2 ± 0.6	23.0 ± 0.6	37.1 ± 2.3	29.0 ± 0.7	52.7 ± 1.3	28.1 ± 0.6	52.7 ± 0.5
C17:1 FAME	62.7 ± 1.2	100 ± 4	93.7 ± 2.7	107 ± 3	94.3 ± 1.9	134 ± 2	93.2 ± 3.3	133 ± 2
C20:1 FAME	223 ± 7	609 ± 16	432 ± 11	461 ± 20	475 ± 5	674 ± 18	442 ± 5	643 ± 15
C22:1 FAME	4.1 ± 0.1	12.1 ± 0.1	7.7 ± 0.0	8.0 ± 0.5	9.3 ± 0.2	16.3 ± 0.3	7.4 ± 0.1	12.5 ± 0.4
C20:2 FAME	53.1 ± 1.7	29.2 ± 0.8	47.3 ± 0.7	32.8 ± 1.0	45.4 ± 0.6	20.1 ± 1.2	55.6 ± 0.8	33.2 ± 0.4
C18:2 FFA	2785 ± 231	3828 ± 360	2519 ± 300	2790 ± 352	2316 ± 161	3341 ± 766	2190 ± 63	3396 ± 422
farnesol	18.1 ± 0.3	124 ± 12	46.9 ± 3.9	61.5 ± 19.0	54.7 ± 3.5	87.6 ± 5.6	50.3 ± 2.6	71.0 ± 4.2
phytol	27.2 ± 1.1	68.2 ± 2.5	40.2 ± 0.7	56.6 ± 4.4	57.4 ± 3.5	95.3 ± 7.3	49.2 ± 1.6	55.9 ± 1.7

[a] The phytic acid contents are expressed in mg/g dry matter. All other metabolites are expressed as relative peak intensities, i.e. metabolite peak intensity/(internal standard peak intensity/100). [b] Values represent means ± standard deviations resulting from the analysis of three aliquots of freeze-dried soybean flour.

Table S6. Differences in metabolites in homozygous wild-type (HWT) und homozygous *lpa* mutant (HM) progenies depending on generations (F_3 vs. F_5) and growing seasons (spring 2014 vs. autumn 2015) ($p < 0.05$).[a,b]

	HWT progenies			HM progenies		
	F_3	$F_{5\text{-}1}$	$F_{5\text{-}2}$	F_3	$F_{5\text{-}1}$	$F_{5\text{-}2}$
	spring 2014	autumn 2015	autumn 2015	spring 2014	autumn 2015	autumn 2015
C11:0 FAME	4.5 ± 0.6	1.0 ± 0.1	0.7 ± 0.1	5.2 ± 0.1	3.0 ± 0.2	2.0 ± 0.8
C14:0 FAME	185 ± 4	144 ± 2	168 ± 4	168 ± 4	142 ± 0	153 ± 1
C15:0 FAME	21.8 ± 0.8	15.1 ± 0.3	17.4 ± 1.2	23.7 ± 0.7	16.6 ± 0.3	16.4 ± 0.8
C26:0 FAME	23.0 ± 0.6	29.0 ± 0.7	28.1 ± 0.6	37.1 ± 2.3	52.7 ± 1.3	52.7 ± 0.5
C15:1 FAME	16.0 ± 0.4	11.8 ± 0.1	14.4 ± 0.6	23.0 ± 0.8	10.3 ± 0.1	15.2 ± 0.5
C16:2 FAME	4.1 ± 0.1	2.9 ± 0.0	3.7 ± 0.1	3.9 ± 0.0	2.0 ± 0.1	2.7 ± 0.1
Δ^7-stigmastenol	17.0 ± 0.3	37.4 ± 2.8	33.4 ± 1.5	16.7 ± 0.6	34.4 ± 0.6	34.0 ± 2.7
24-MCA	4.3 ± 0.0	7.6 ± 0.4	7.6 ± 0.2	3.2 ± 0.1	5.5 ± 0.5	5.0 ± 0.2
campesterol	92.3 ± 2.3	133 ± 4	168 ± 6	88.3 ± 3.0	140 ± 4	130 ± 7
cholesterol	1.9 ± 0.1	3.7 ± 0.2	4.2 ± 0.3	1.6 ± 0.1	2.8 ± 0.5	3.4 ± 0.3
stigmasterol	114 ± 2	184 ± 8	197 ± 8	149 ± 3	236 ± 13	248 ± 17
β-sitosterol	305 ± 1	420 ± 17	489 ± 26	298 ± 7	420 ± 16	427 ± 24
δ-tocopherol	45.9 ± 1.8	75.4 ± 3.5	71.6 ± 3.6	36.4 ± 1.4	52.1 ± 7.7	54.4 ± 3.1
arabitol	1.9 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	2.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.0
glycerol	17.5 ± 1.4	4.0 ± 0.1	4.5 ± 0.1	16.7 ± 0.6	5.5 ± 0.2	6.5 ± 0.0
mannitol	5.7 ± 0.2	1.3 ± 0.0	2.3 ± 0.1	3.8 ± 0.3	1.8 ± 0.0	2.1 ± 0.1
pintiol	332 ± 5	138 ± 3	161 ± 10	311 ± 12	249 ± 8	265 ± 8
ribitol	0.9 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.8 ± 0.0
sorbitol	16.4 ± 0.5	1.8 ± 0.1	2.1 ± 0.2	19.6 ± 0.4	3.5 ± 0.1	3.5 ± 0.1
fructose	3.8 ± 0.1	1.5 ± 0.1	2.0 ± 0.6	4.7 ± 0.1	1.6 ± 0.1	2.0 ± 0.0
raffinose	398 ± 12	350 ± 8	323 ± 9	153 ± 4	129 ± 2	128 ± 1
cysteine	0.7 ± 0.2	1.8 ± 0.1	1.7 ± 0.1	1.3 ± 0.2	2.5 ± 0.1	2.5 ± 0.2
glutamic acid	182 ± 23	244 ± 2	263 ± 6	273 ± 19	432 ± 10	382 ± 6
lysine	8.0 ± 1.9	1.6 ± 0.2	1.9 ± 0.2	4.3 ± 1.0	0.7 ± 0.2	1.3 ± 0.2
tyrosine	21.1 ± 0.1	15.0 ± 0.3	14.1 ± 0.8	20.7 ± 0.8	9.3 ± 0.2	11.1 ± 1.0
norvaline	3.4 ± 0.7	1.3 ± 0.1	1.2 ± 0.1	4.1 ± 0.5	1.6 ± 0.0	1.8 ± 0.1
ethanolamine	25.6 ± 6.5	4.6 ± 0.2	7.7 ± 0.6	18.6 ± 4.1	7.8 ± 1.7	8.1 ± 1.1
p-coumaric acid	1.0 ± 0.2	1.5 ± 0.1	2.2 ± 0.1	1.0 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
malonic acid	19.1 ± 2.1	10.0 ± 0.2	8.6 ± 0.9	35.3 ± 4.9	11.0 ± 0.9	6.8 ± 0.6
pyruvic acid	1.2 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	1.0 ± 0.1	1.9 ± 0.0	1.6 ± 0.2
β-hydroxybutyric acid	0.8 ± 0.1	1.9 ± 0.0	2.1 ± 0.2	0.8 ± 0.1	2.1 ± 0.1	1.9 ± 0.3
γ-hydroxybutyric acid	2.0 ± 0.1	1.2 ± 0.2	1.3 ± 0.0	4.2 ± 0.3	2.2 ± 0.1	2.0 ± 0.1

[a] All metabolites are expressed as relative peak intensities, i.e. metabolite peak intensity/(internal standard peak intensity/100). [b] Values represent means ± standard deviations resulting from the analysis of three aliquots of freeze-dried soybean flour.

7 PUBLICATIONS AND PRESENTATIONS

PEER-REVIEWED PUBLICATIONS

Goßner, S.; Yuan, F.; Zhou, C.; Tan, Y.; Shu, Q.; Engel, K.-H., Stability of the Metabolite Signature Resulting from the *MIPS1* Mutation in *Low Phytic Acid* Soybean (*Glycine max* L. Merr.) Mutants upon Cross-Breeding. *J. Agric. Food Chem*. 2019, *67*(17), 5043-5052.

Goßner, S.; Yuan, F.; Zhou, C.; Tan, Y.; Shu, Q.; Engel, K.-H., Impact of Cross-Breeding of *Low Phytic Acid MIPS1* and *IPK1* Soybean (*Glycine max* L. Merr.) Mutants on their Contents of Inositol Phosphate Isomers. *J. Agric. Food Chem*. 2019, *67*(1), 247-257.

Zhou, C.; Tan, Y.; **Goßner, S**.; Li, Y.; Shu, Q.; Engel, K.-H., Impact of Crossing Parent and Environment on the Metabolite Profiles of Progenies Generated from a *Low Phytic Acid* Rice (*Oryza sativa* L.) Mutant. *J. Agric. Food Chem*. 2019, *67*(8), 2396-2407.

Zhou, C.; Tan, Y.; **Goßner, S**.; Li, Y.; Shu, Q.; Engel, K.-H., Impact of Cross-Breeding of *Low Phytic Acid* Rice (*Oryza sativa* L.) Mutants with Commercial Cultivars on the Phytic Acid Contents. *Eur. Food Res. Technol*. 2019, *245*(3), 707-716.

Zhou, C.; Tan, Y.; **Goßner, S**.; Li, Y.; Shu, Q.; Engel, K.-H., Stability of the Metabolite Signature Resulting from the *OsSULTR3;3* Mutation in *Low Phytic Acid* Rice (*Oryza sativa* L.) Seeds upon Cross-breeding. *J. Agric. Food Chem*. 2018, *66*(35), 9366-9376.

Jockel-Schneider, Y.*; **Goßner, S. K.***; Petersen, N.; Stölzel, P.; Hägele, F.; Schweiggert, R. M.; Haubitz, I.; Eigenthaler, M.; Carle, R.; Schlagenhauf, U., Stimulation of the nitrate-nitrite-NO-metabolism by repeated lettuce juice consumption decreases gingival inflammation in periodontal recall patients: a randomized, double-blinded, placebo-controlled clinical trial. *J. Clin. Periodontol.* 2016, *43*(7), 603-608 (***both authors contributed equally to the work**).

OTHER PUBLICATIONS

Zhou, C.; Tan, Y.; **Goßner, S**.; Li, Y.; Shu, Q.; Engel, K.-H., Impact of Cross-Breeding on the Metabolites of the *Low Phytic Acid* Rice Mutant *Os-lpa*-MH86-1. Proceedings of the FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology, August 27-31, 2018, Vienna, Austria (in print).

Goßner, S. K.; Pixa, M.; Hinrichs, J., Fraktionierung von Milchfett: Verfahren, Prinzipien und Potenziale. *Molkerei Industrie. Fachmagazin für Milchverarbeitung.* Teil I: 12/2014, S. 15-17.
Teil II: 01/2015, S. 18-19.

POSTER PRESENTATIONS

Goßner, S.; Zhou, C.; Yuan, F.; Shu, Q.; Engel, K.-H., Influence of Cross-Breeding on the Phytic Acid Contents of *Low Phytic Acid* Rice and Soybean Mutants. FAO/IAEA International Symposium on Plant Mutation Breeding and Biotechnology, August 27-31, 2018, Vienna, Austria.

Goßner, S.; Tan, Y.; Yuan, F.; Shu, Q.; Engel, K.-H., Characterization of *low phytic acid* (*lpa*) soybean mutants and their crossbreds by means of metabolite profiling. Max Rubner Conference 2016 - Food Metabolomics, October 10-12, 2016, Karlsruhe, Germany.