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Metabolite Profiling – A Useful Tool for the Investigation of Induced Mutants and Developmental Changes in Crops

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TABLE OF CONTENTS

Abbreviations

ATP	Adenosintriphosphate
CI	Confidence interval
CV	Column volume
DNA	Deoxyribonucleic acid
DW	Dry weight
EI	Electron impact ionization
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization of the United Nations
FID	Flame ionization detection
g	Gram
GC	Gas chromatography
GM	Genetically modified
h	Hour
ha	Hectare
HPLC	High performance liquid chromatography
I.D.	Inner diameter
ILSI	International Life Sciences Institute
InsP	Inositol phosphate
KI	Kovats indices
LDPE	Low-density polyethylene
LG	Long grain
min	Minute
ml	Milliliter
mm	Millimeter
MS	Mass spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacteamide
MTBE	Methyl-*tert*-butylether
mu	Mass units
NIR	Near infrared
NMR	Nuclear magnetic resonance
OECD	Organization for Economic Co-operation and Development

PC	Principal component
PCA	Principal component analysis
RI	Refraction index
RT	Retention time
s	Second
SD	Standard deviation
SG	Short grain
SPE	Solid phase extraction
TIC	Total ion count
TMS	Trimethylsilyl-
TMSIM	Trimethylsilylimidazole
UPLC	Ultra performance liquid chromatography
WHO	World Health Organization

1 INTRODUCTION AND OBJECTIVES

Metabolites are the end products of cellular processes and represent the ultimate reflection of the response of biological systems to genetic or environmental changes [1]. “Unbiased” approaches to metabolite analysis have been developed in recent years providing tools that complement other untargeted techniques, such as transcriptomics or proteomics [2]. Metabolomics, i.e. the measurement of all metabolites in systems under given conditions, is an extremely challenging goal requiring the interplay of various complementary techniques. Metabolite profiling can be considered as one of the most pragmatic approaches presently applied. It aspires to provide a comprehensive picture by extracting, detecting, identifying and quantifying a broad spectrum of the metabolites present in complex biological systems in an effective and reproducible way and thus to get a deeper insight into these systems [1, 3, 4]. Among the various technology platforms established for metabolite profiling, the coupling of capillary gas chromatography and mass spectrometry (GC-MS) proved to be one of the most robust methodologies, in particular for a comprehensive analysis of primary plant metabolites [5].

Metabolite profiling is being discussed as additional tool for the safety assessment of genetically modified crops because of its potential to increase the probability to detect unintended effects [6, 7]. In addition to the application for safety assessment, metabolite profiling was suggested to provide valuable data for breeding-driven metabolic engineering [8, 9] and as approach to extend and to enhance the power of functional genomics [4]. During the past decade, metabolite profiling techniques have been applied to the assessment of phenotypic diversity in plants [10] and to comparative investigations of breeding systems, e.g. conventional versus genetically modified crops [11], farming practices [12] and environmental impacts [13]. In addition, plant developmental systems have been investigated by means of metabolite profiling [14-17]. One example for an important stage in the development of plants is the germination of seeds. This phase in the life cycle of a plant is characterized by a combination of various catabolic and anabolic processes. Distinct and time-dependent alterations in metabolite levels are to be expected and metabolite profiling should be a suitable analytical tool to provide a comprehensive picture of these changes.

The aim of the present work was to investigate the usefulness of metabolite profiling approach based on GC-MS for an unbiased analysis of various plant systems. In the first part, the suitability of the metabolite profiling method should be demonstrated by following metabolic changes in plant seeds in the course of germination. Germination represents an important stage in the development of plants and seeds. In addition, germination processing has the potential to improve the nutritional value of crops. Brown rice seeds and barley kernels were selected as model crops for the investigation of germination time-related metabolic changes. The objectives were to analyze a broad spectrum of low molecular weight compounds covering a wide range of chemical classes in the germinating crop materials and to follow their dynamic changes via univariate and multivariate analytical methods.

In addition to the investigation of developmental plant systems, the applicability of metabolite profiling for the detection of changes in the metabolite phenotype induced by mutation breeding should be tested. Therefore, the metabolic profiles of low phytic acid (*lpa*) mutants of rice and soybean, generated through γ-irradiation, should be compared to those of the corresponding wild-types in order to explore the approach for the assistance in the elucidation of different types of mutation which underly the specific phenotype. Moreover, the applied metabolite profiling should be used for the assessment of metabolic differences between *lpa* mutants and wild-types in the light of environmental impacts on the crop metabolite phenotype. To complement the unbiased metabolite profiling of these low phytic acid mutants, the analysis of nutritionally relevant minerals should be included into the investigation of *lpa* rice and soybean to assess potential effects of *lpa* mutations on the levels of minerals.

2 BACKGROUND

2.1 CROPS IN AGRICULTURE AND NUTRITION

2.1.1 Rice, barley and soybean

Rice as staple food is one of the most important cereal crops worldwide with a paddy rice production of nearly 652 Mio. tons in 2007 [18]. It provides 20 % of the worlds dietary energy supply [19]. 90% of the world rice production originates from Asia, mainly China and India. Within the European Union (EU), Italy and Spain are the major producers of paddy rice [18]. The two major species of rice are *Oryza sativa*, mainly cultivated in Asia, and *Oryza glaberrima* which is grown in Africa. Rice can be differentiated into small (e.g. *Oryza sativa* ssp. *japonica*) and long grain (e.g. *Oryza sativa* ssp. *indica*) subspecies. Rice is the second plant species for which the complete genome has been determined in 2004 by the International Rice Genome Sequencing Project and it thus represents a major model plant in basic and applied research [20].

Barley has a long history as a domesticated crop. It has been one of the first crops cultivated in agriculture [21]. Major production countries for cultivated barley (*Hordeum vulgare*) are Russia (15.6 Mt./a), Canada (11.8 Mt./a) and Germany (11.0 Mt./a). The worldwide production accounted to 136 Mt. in 2007 [18]. Barley grains are predominantly used in the brewing industry and in the production of animal feed [21].

Soybean (*Glycine max* L. Merr) is the most important legume worldwide with a production of 216 Mt./a [18]. It is the major oil seed in world trade accounting for approximately 56% of the global oilseed production [22]. Due to high contents of nutritionally important constituents, e.g. essential amino acids and unsaturated fatty acids, soybeans are widely used as raw material of processed foods. Commonly known soybean-derived products are tofu, miso and soy sauce. Moreover, soybean oil is the major source for the extraction of lecithin [23].

2.1.2 Germinated brown rice

In human nutrition, rice is mainly consumed in milled form. However, milled rice is poor regarding nutritionally relevant macro- and micronutrients, e.g. amino acids, vitamins and minerals. Brown rice, which includes bran and germ, contains more nutritionally relevant constituents compared to milled rice [24]. However, it is still less popular because of its dark appearance and hard texture [25, 26]. Germination of brown rice has been considered to overcome this problem by making the rice easier to prepare and to chew. Moreover, germinated brown rice contains more macro- and micronutrients than ordinary brown rice [25].

Proximate analysis of germinated brown rice revealed an increase in the levels of dietary fiber, reducing sugars, free amino acids, vitamins and minerals [27-32]. In addition, increased contents of the nutritionally important constituents γ-oryzanol, tocotrienols, ferulic acid and inositol have been reported [29, 33, 34]). γ-Aminobutyric acid (GABA), a well known inhibitory neurotransmitter, was shown to be significantly increased in germinated brown rice compared to ordinary brown rice [25, 34-36] Further, the level of phytic acid, a major anti-nutrient in rice, was shown to decrease during germination [37].

Germinated brown rice is commercially available in Japan since 1995 [26]. Various food products containing germinated brown rice, e.g. rice balls, soup, bread and burgers have been developed [25, 38]. In addition to brown rice, germination of other staple food, e.g. wheat, soybean, beans, lentils and peas have been suggested for further improvement of their nutritional value [39-42].

Based on the described increased levels of nutritionally important rice constituents, several health effects have been observed after the intake of germinated brown rice products. Consumption of pre-germinated brown rice meals was reported to contribute to decreased blood glucose levels [43], to inhibit cancer cell proliferation [44], to prevent Alzheimer´s disease [45] and to have beneficial effects on psychosomatic health [46].

2.2 PHYTIC ACID

2.2.1 Structure and natural occurrence

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate or $InsP_6$) is the major storage form of phosphorus in mature seeds or grains. Its structure is shown in Figure 1. Typically, between 65 and 85 % of seed total phosphorus is found in this compound [47].

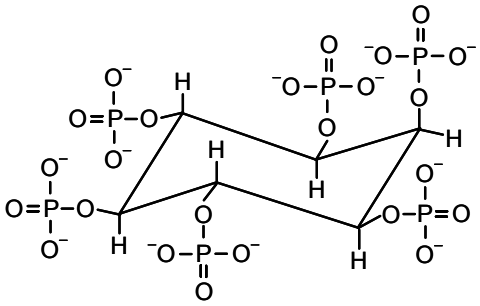

Figure 1: Structure of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate)

In beans phytic acid contents up to 9 % have been described [48]. The contents in cereals vary from 0.6 to 2.2 %; amounts reported in barley and brown rice range from 0.4 to 1.2 % and 0.6 to 1.1 %, respectively [48, 49]. For soybeans, ranges between 1.0 and 2.2 % have been reported [48]. Phytic acid in plants occurs primarily as mixed salts of mono- and divalent cations, particularly K and Mg, together with minor amounts of Ca, Fe, Zn and Mn [47]. Phytates, the salts of phytic acid, are deposited as globular inclusions called globoids. They are located within the protein storage vacuoles in the aleurone and the germ of crops. For rice and barley, approximately 80% of the mixed phytic acid salts are found in the aleurone. In soybeans, phytate deposits are dispersed throughout the germ and cotyledonary tissues [50]. During crop ripening and maturation, phytic acid accumulates in the grains and seeds and reaches the highest level at the stage of maturity [48]. In the course of germination, phytate salts are broken down by phytases which releases phosphorus, phytic acid-bound minerals and *myo*-inositol needed by the growing seedling [51].

2.2.2 Biosynthesis of phytic acid

The biochemical pathways leading to phytic acid are shown in Figure 2. The first step in the biosynthesis of phytic acid is the conversion of D-glucose 6-phosphate to 1D-*myo*-inositol 3-phosphate ($Ins(3)P_1$) catalyzed by 1D-*myo*-inositol 3-phosphate synthase (MIPS) [52, 53]. The subsequent routes leading to phytic acid are not fully clarified. A sequential phosphorylation of $Ins(3)P_1$ to phytic acid was proposed for seed germination and development [54]. The mechanism of a stepwise phosphorylation of $Ins(3)P_1$ to $Ins(1,2,3,4,5,6)P_6$ is supported by the identification of the specific inositol phosphate kinases (InsPK) [55, 56]. In addition, recombinant InsPK cloned from rice (*OsIpk*) and barley (*HvIpk*) were shown to have multiple activities for a broad range of *myo*-inositol phosphate intermediates [57].

The conversion of D-glucose 6-phosphate to $Ins(3)P_1$ is also the first committed step in the *myo*-inositol biosynthesis [58]. $Ins(3)P_1$ is converted to free *myo*-inositol and inorganic phosphorus by means of a *myo*-inositol monophosphatase (MIP). Free *myo*-inositol might also act as source for the biosynthesis of phytic acid via *myo*-inositol kinase (MIK) enzyme activity. A MIK has been isolated from maize which phosphorylates *myo*-inositol but not $Ins(3)P_1$ [59].

In addition to the above-mentioned lipid-independent pathway, the biosynthesis of phytic acid might also start from *myo*-inositol via a lipid-dependent pathway (Figure 2). This pathway involves the formation of several phosphatidylinositol phosphate intermediates. Initially, it begins with the conversion of *myo*-inositol and phosphatidic acid to phosphatidylinositol (PtdIns), catalyzed by a phosphatidylinositol synthase (PtdInsS). PtdIns and phosphatidylinositolphosphate intermediates ($PtdIns(4)P_1$, $PtdIns(4,5)P_2$) are phosphorylated via kinase activities (PtdInsK, PtdInsPK). A kinase and phospholipase C catalyzed reaction on $PtdIns(4,5)P_2$ results in diacylglycerol and $Ins(1,4,5)P_3$, which is then further phosphorylated, comparable to the lipid-independent pathway [60, 61].

Inositol phosphates with a higher degree of phosphorylation than $InsP_6$, e.g. $InsP_7$ and $InsP_8$, have been detected in eukaryotic cells [62, 63]. However, the biosynthesis of highly phosphorylated InsP in plants is still not well understood.

Degradation of phytic acid by means of the enzyme phytase, naturally present in plants and certain microorganisms, occurs during various plant developmental

stages. Additionally, various food processing procedures like steeping, germination, malting and fermentation may lead to phytic acid degradation [64].

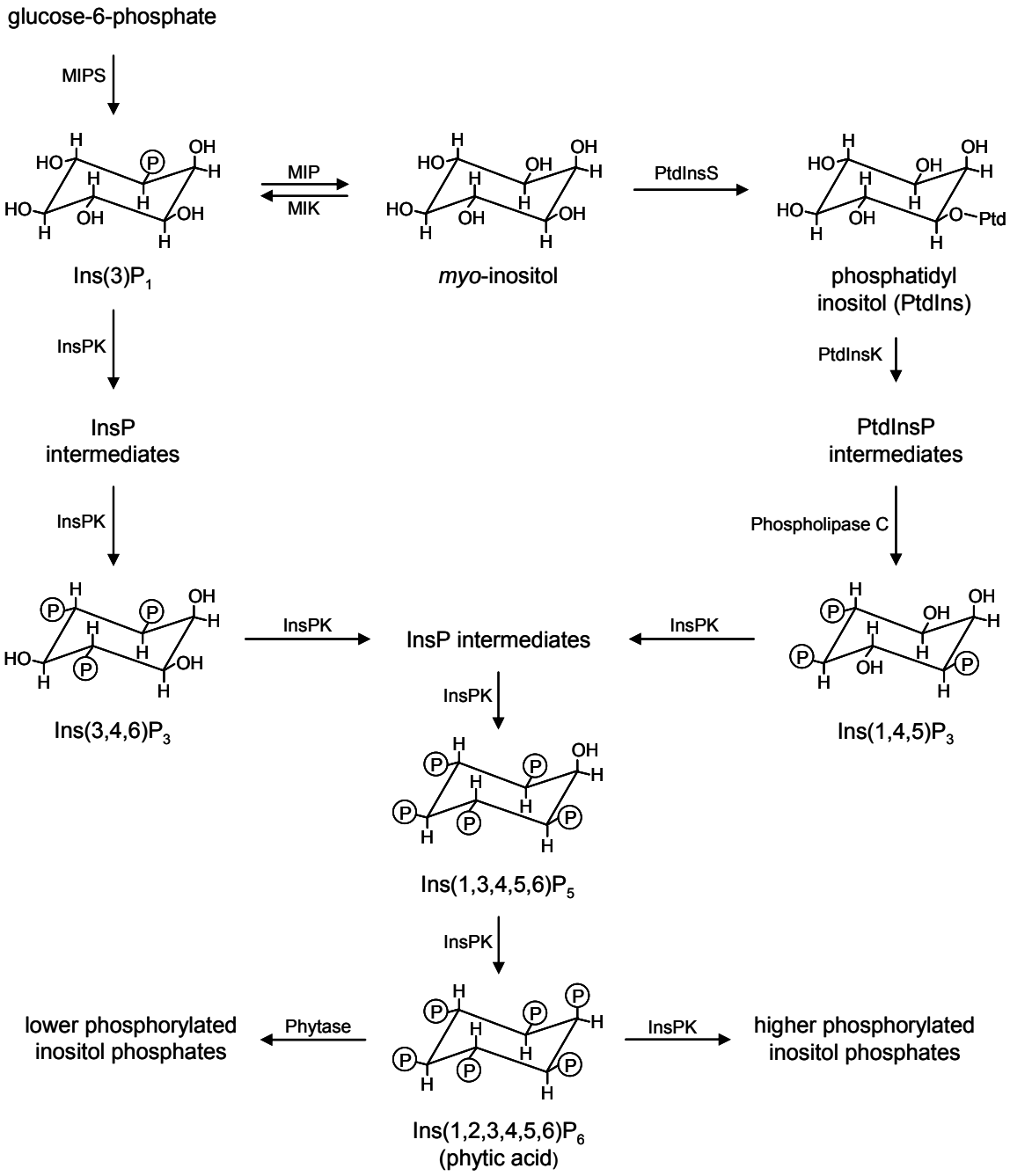

Figure 2: Plant biosynthetic pathways leading to phytic acid [60, 61]. MIPS, *myo* inositol phosphate synthase; MIP, *myo*-inositol monophosphatase; MIK, *myo*-inositol kinase; PtdInsS, phosphatidylinositol synthase; InsPK, inositolphosphate kinase; PtdInsK, phosphatidylinositol kinase.

2.2.3 Relevance in agriculture and nutrition

Approximately 75 % of the total seed phosphorus is stored in form of phytic acid depending on crop cultivar, soil conditions and fertilizer applications [47]. An amount of 35 Mio. tons phytic acid, containing 9.9 Mio. tons phosphorus has been estimated for a worldwide production of 4.1 billion tons of crop seeds and fruits. The amount of phosphorus corresponds to 65 % of the globally traded elemental phosphorus used in mineral fertilizers [65].

Phytic acid is poorly degraded in the digestive system of non-ruminants making the contained phosphorus unavailable for animals [66]. Therefore, animal feed producers and farmers add inorganic phosphorus to the feed. However, this leads to an excess of phosphorus in the animals waste, which is considered as major source of phosphorus pollution from agricultural sources. Moreover, phytic acid, excreted in manure, is degraded by natural soil microorganisms releasing phosphorus which contributes to an eutrophication of water [67].

Phytic acid is widely considered as an anti-nutrient in food and feed. It is stored as mixed salts of cations, mainly potassium and magnesium and it limits the bioavailability of nutritionally relevant minerals such as calcium, iron, zinc and selenium by formation of indigestible chelates [68-70]. This represents a problem especially in developing countries where people rely on staple cereals and legumes which contain noteworthy amounts of phytic acid [71]. Estimated phytate contents in human diets were 600 – 800 mg/ day in the industrial countries USA and the UK, whereas it was found to be significantly higher in developing countries with a lower meat consumption, e.g. Nigeria (on average: 2100 mg/day). For example, lowered absorption of iron and zinc from diets rich in phytic acid is discussed as a major factor for iron and zinc deficiencies in developing countries [71].

Phytic acid has the ability to react with negatively charged groups of proteins and may therefore have adverse effects on protein digestion and bioavailability [72, 73]. In addition, the presence of phytate resulted in a significantly reduced rate of starch digestion *in vitro* [74].

However, even if phytic acid is widely considered as anti-nutrient, potential beneficial effects have also been reported. The role of phytic acid as antioxidant, in reducing the cancer risk and in preventing heart disease in animal studies has been extensively reviewed in literature [75-80].

2.2.4 Generation of low phytic acid (*lpa*) crops

To overcome mineral deficiencies in human and animal nutrition, various efforts have been made to generate nutritionally improved crops either by mineral biofortification or by lowering the contents of mineral-binding phytic acid. Biofortified staple crops can be produced by conventional breeding strategies [81-83] or by genetic engineering [84]. Other approaches are based on the reduction of phytic acid contents in staple crops.

A possibility to reduce the phytic acid content is the addition of exogenous phytase to food and feed [85, 86]. More recently conducted approaches aim to suppress the biosynthesis of phytic acid during the crop development. Genetic engineering has been successfully applied to produce low phytic acid (*lpa*) crops of rice, maize and soybean [87-89]. Alternatively to the production of transgenic plants, the application of mutation breeding, considered as a conventional breeding method, has been used to develop crops with lowered phytic acid contents. Mutation breeding has a long history since the first induced mutation experiments on barley in 1928 [90]. Meanwhile, a total of about 3000 mutant crop varieties had been developed [91]. Regarding the mutation breeding of low phytic acid mutants, the use of γ-irradiation [92, 93] and chemically induced mutagenesis [94, 95] have been described for the development of various *lpa* cereals and legumes including maize [94-96], barley [97, 98], rice [92, 99, 100], wheat [101] and soybean [93, 102, 103]. These *lpa* mutants exhibit significantly reduced phytic acid contents ranging from -50 % to more than -95 % compared to wild-types.

The decrease in phytic acid is typically accompanied by increased contents of inorganic phosphorus, whereas the total phosphorus contents remain almost unchanged in *lpa* mutants and wild-types Therefore, physically or chemically treated *lpa* seeds are screened and selected via an assay based on the determination of inorganic phosphorus. A simplified flow chart of the production of *lpa* crops by means of γ-irradiation is shown in Figure 3.

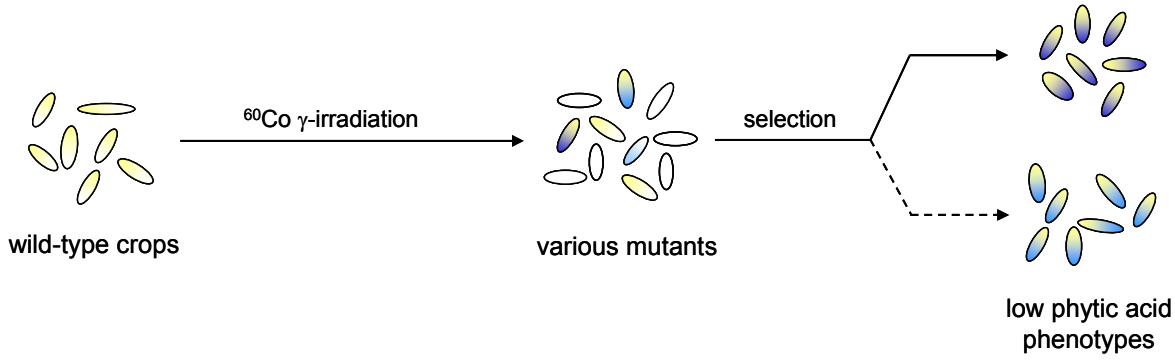

Figure 3: Production of low phytic acid crops by means of γ-irradiation.

The process of mutation breeding is quite extensive and laborious. For example, several ten thousand M2 plants of rice and soybean, derived from the irradiation-treated wild-types (M1 seeds), were grown in fields [93, 99]. Seeds, harvested from these M2 plants were used for the selection process based on the determination of inorganic phosphorus.

To date, a main focus in the area of *lpa* crop research is on the agronomic properties of these mutants. Although *lpa* mutants were shown to be nutritionally improved, agronomic parameters like plant growth, yield and stress tolerance might be negatively affected [60, 104, 105].

2.2.5 Methods for determination of phytic acid

During the past century, various techniques have been developed for the determination of phytic acid including chromatographic and spectroscopic methods. First quantitative methods for the determination of phytic acid were based on a precipitation reaction with ferric chloride [106]. However, a major drawback of these methods is their low sensitivity [107]. The ion-pair high performance liquid chromatography (ion-pair HPLC) represents a robust and suitable method for the separation and detection of phytic acid and lower inositol phosphates [108, 109]. In principle, it is based on the binding of the negatively charged phytic acid to tetrabutyl ammonium cations, which can bind to the non-polar stationary phase of the HPLC column.

In addition to chromatographic approaches, spectroscopic methods have been used to quantify phytic acid in crops and food. A major advantage of the nuclear magnetic resonance (NMR) spectroscopy [110] is the ability to determine the position of the phosphate groups within the different inositol phosphates which allows a separation according to structural isomers. However, drawbacks of this technique are the expensive equipment and its low sensitivity making it less useful for the detection of low amounts of inositol phosphates [107].

2.3 METABOLOMICS

2.3.1 Definitions

Unbiased profiling techniques, so called Omics-techniques, are being discussed as powerful tools in the field of functional genomics such as DNA sequencing, transcription profiling, protein and metabolite analyses [111]. Ultimate objectives of the different Omics-techniques are to discover the complete genome (genomics), transcriptome (transcriptomics), proteome (proteomics) and metabolome (metabolomics) in a biological system.

The measurement of nucleotide sequences of the genome is the first step within the investigation of the phenotype. Transcriptomics, i.e. the detection and identification of genes being activated (expressed as messenger RNA) in a biological system at different times under different environmental conditions, represents the link between the analysis of genotype and phenotype and is the basis for functional genomics. A parallel screening of a large number of gene sequences for the detection of differences in gene expression in plant tissues can be achieved by means of DNA microarray technology [112]. Proteomics comprises the measurement of all proteins in a biological system under the defined conditions. The method of choice used for the proteome analysis is based on two-dimensional gel electrophoresis (2DGE). The resulting protein spots are excised from the gel, digested by specific proteases and the proteins are analyzed by means of MS [113]. Regarding the proteomics analysis, it has to be considered, that the actual proteome cannot be predicted from the transcriptome because of post-translational modifications, e.g. glycosylation and phosphorylation.

Metabolomics, that is the measurement of all metabolites in biological systems under given conditions, is an extremely comprehensive challenge. The metabolites represent the end products of the interactions between the genome, transcriptome and proteome with the environment and thus define the biochemical phenotype of a system [114].

2.3.2 Metabolite Profiling

The two major techniques which are being applied to perform metabolomics-based investigations are metabolic fingerprinting and metabolite profiling. Metabolic fingerprinting represents a rapid screening method for biological samples without a major pretreatment of the material to be analyzed. In order to screen a large number of these samples, e.g. in plant breeding programs, it might not be necessary to determine the individual level of every metabolite. Instead, a rapid classification of samples according to their biological relevance might be sufficient. Methods like nuclear magnetic resonance (NMR), Fourier transform infrared (FT-IR) spectroscopy and mass spectrometry (MS) are used within this approach [1].
Metabolite profiling represents the analysis of selected compounds from the same chemical compound classes or compounds linked by known metabolic relationships. Metabolite profiling can be considered as one of the most pragmatic approaches presently applied. It aims at the detection, identification and quantification of a broad spectrum of compounds in a single sample in an effective and reproducible way to provide a deeper insight into the complex biological system [1, 3, 4]. The metabolite profiling procedure can be subdivided into the following principle steps:

- Sampling of raw material and sample preparation
- Extraction (and fractionation) of metabolites
- Analysis of metabolites
- Data processing and statistical analysis

Prior to extraction of the metabolites, it has to be made sure that all metabolic processes in the plant material are rapidly stopped. Freezing the material in liquid nitrogen and subsequent freeze-drying has often been applied within the metabolite profiling sample preparation [1, 115, 116]. For the extraction of metabolites, pure organic solvents or mixtures of solvents are added to the milled plant samples. Polar metabolites are usually extracted with methanol, ethanol and water, whereas chloroform, chloroform / methanol mixtures or dichloromethane have been used for the extraction of lipophilic compounds from the biological matrix [1, 117]. An extraction and fractionation scheme developed for the metabolite profiling of rice is shown in Figure 4 [118]. The approach, based on consecutive extraction of lipids and polar compounds, is comparable to other profiling strategies with respect to the extraction of metabolites with solvents differing in polarity [115, 119]. However,

additional transesterification-solid phase extraction (lipids) and selective hydrolysis of silylated derivatives (polar compounds) were applied to separate major from minor constituents. This procedure results in four fractions containing fatty acid methyl esters and hydrocarbons (fraction I), free fatty acids, alcohols and sterols (fraction II), sugars and sugar alcohols (fraction III), acids, amino acids and amines (fraction IV).

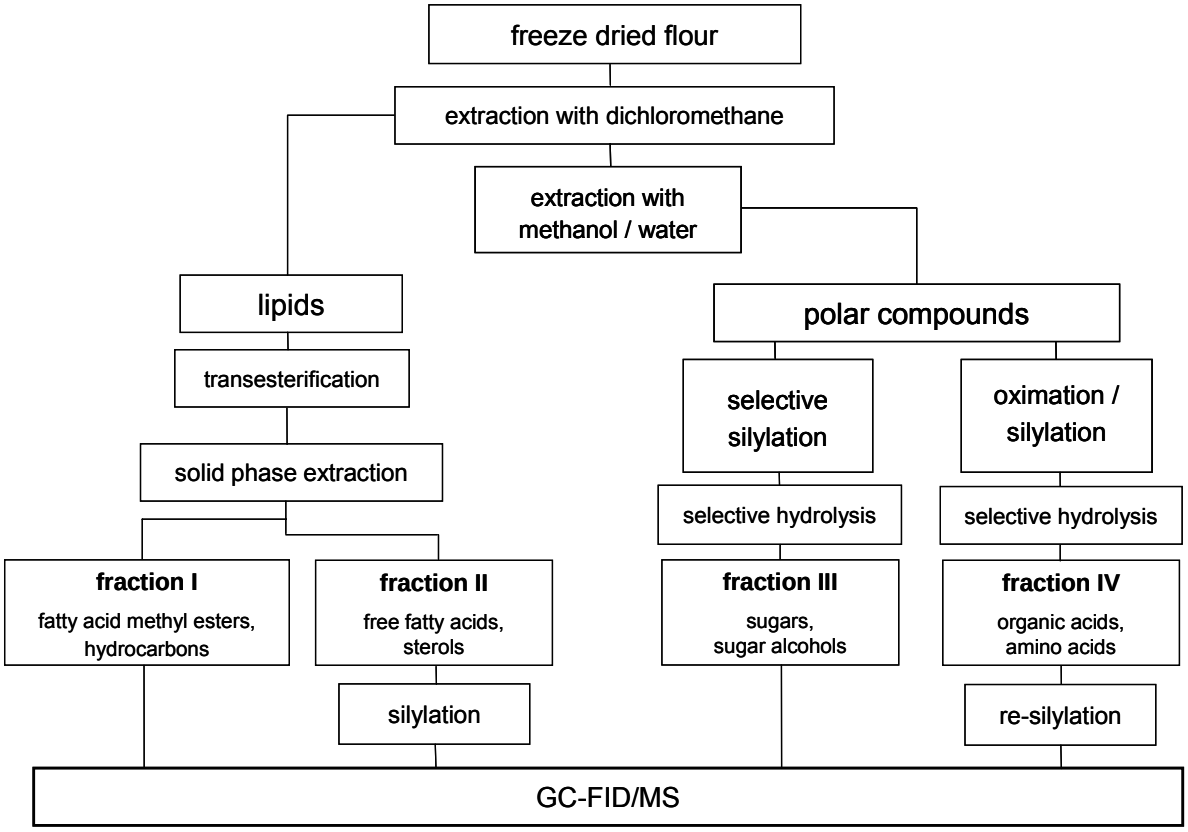

Figure 4: Extraction and fractionation scheme of freeze-dried rice flour [118]

For the analysis of metabolites, different technology platforms have been established. Coupling of liquid or capillary gas chromatography and mass spectrometry (LC-MS, GC-MS) have proven to be the most applied methodologies within the scope of metabolomics-based investigations [5, 117, 120-122]. Newer GC-time-of-flight (TOF)-MS systems combine a high separation efficiency and resolution of capillary GC with the high sensitivity of mass-selective detection making it attractive in GC-based metabolome analysis. However, owing to inherent features of the approach, e.g. choice of extraction solvents, derivatization steps or volatilities of derivatives, it has to be considered that the type of metabolites covered is to some extent pre-determined and not fully “unbiased”. Therefore, based on the assumption that plants may contain

potentially thousands of metabolites, the use of a single GC technique still has its limitations. Therefore, the application of multi-dimensional gas chromatography or platform crossing techniques might help to improve the detection and identification of plant metabolites and qualitative and quantitative metabolite profiling of plant systems. A promising approach is the application of two-dimensional gas chromatography combined with time-of-flight mass spectrometry (GC x GC-TOF-MS). This approach allowed, for example, the detection of more than 1400 peaks in a single chromatogram obtained from a basil extract [123].

Beside the use of GC, the LC-based investigation of metabolites is an aspiring field in metabolomics [124]. In contrast to GC-MS analysis, fewer sample preparation steps (e.g. derivatization) are necessary. Moreover, LC-MS can be adapted to a wider array of substances, including a range of secondary metabolites such as alkaloids, flavonoids, glucosinolates, isoprenes and saponins [125]. High-performance LC (HPLC) is used as standard separation technique prior to MS analysis. For example, LC coupled to MS has been used for the phenotyping of plants [126] and to follow metabolic changes in fermented food samples [127]. However, this technique has some drawbacks in the chromatographic performance compared to GC. The use of ultra-performance LC (UPLC) might overcome these drawbacks by increasing the chromatographic resolution and allowing a more rapid analysis in functional genomic studies [117, 128].

The application of profiling techniques to a multitude of biological samples results in a huge amount of metabolite-related data. In order to analyze and compare those data, statistical analytical methods such as principal component analysis (PCA), hierachical cluster analysis (HCA) and self-organizing maps (SOMs) proved to be useful tools in the analysis and evaluation of metabolite profiling data [11, 114, 123]. Analysis of polar metabolites obtained from GC-MS profiling of wild-type and transgenic potato genotypes by means of PCA and HCA resulted in a clustering of the wild-type and transgenic genotypes [11]. A principal component analysis of GC x GC-Tof-MS data obtained from basil, peppermint and stevia revealed a distinct separation between the different plant species [123].

2.3.3 Metabolomics-based Applications

Due to the unbiased character of metabolomics-based profiling methods, they have been suggested to provide valuable information in various fields of plant / food analysis including safety assessment of GM-derived and novel foods, food quality / authenticity assessment, plant / crop phenotyping and plant functional genomics [7, 117, 122, 129, 130].

2.3.3.1 Safety assessment of novel foods

Before introducing novel foods to the EU market, comprehensive safety assessments are required [131]. A key element in the safety assessment of novel foods, originally introduced for the assessment of GM-derived food in the beginning of the 1990s by the Organisation of Economic Co-operation and Development (OECD) [132] is the concept of “substantial equivalence”. Although this concept was introduced for GM foods, it has been also recommended for the assessment of non-GM novel foods [133]. It is based on the idea, that if a novel food is found to be substantially equivalent to an existing food, it can be treated in the same manner with respect to safety as the commonly accepted conventional comparator. If the novel food is equivalent to the conventional counterpart except for some well defined differences, that means partially equivalent, the safety assessment will be targeted focusing on these differences. Novel foods, which were classified as non-equivalent to the conventional comparator as a result of multiple differences or due to the fact that there is no adequate comparator available, require extensive safety assessments. Those procedures for novel foods (GM and non-GM derived) have been extensively reviewed in literature [7, 134-136].

It has to be considered, that the above mentioned concept of substantial equivalence represents only the starting point in the safety assessment process on the basis of which the decision on further assessment procedures has to be made. Briefly, the concept is used to identify similarities and differences between the novel food and a conventional comparator and includes a comprehensive characterization of the novel food, e.g. molecular analysis, agronomic performance and chemical composition (Figure 5).

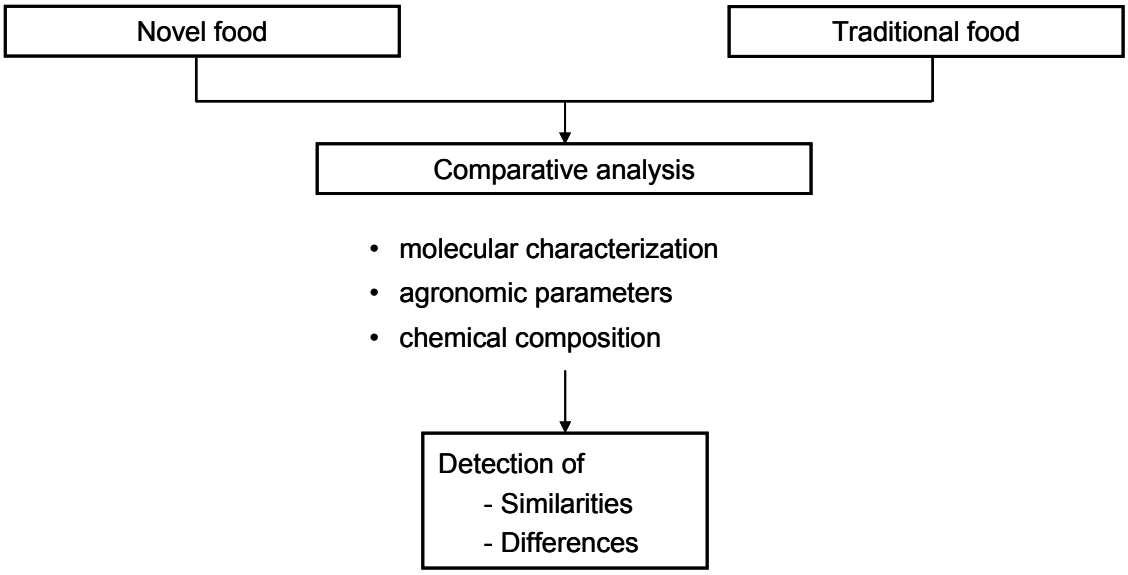

Figure 5: Scheme of a comparative safety assessment of novel foods

In order to specify conventional or traditional foods, which are used for the comparison with novel foods, the term “history of safe (food) use” has been introduced into the safety assessment process of novel foods. To date, there exists no consensus document describing whether an existing food or crop may be considered to have a history of safe use. Only few definitions on the term “history of safe use” have been published so far [137-139].

For GM-derived foods, parental lines accepted to have a history of safe use, can usually be applied as conventional comparators within a safety assessment. Regarding the assessment of non-GM derived novel foods in the EU, the situation is more complicated. A comparable accepted traditional food with a proven history of safe use must be found or the novel food has to be verified to have itself a history of safe use in a country outside the European Union. However, traditional food, considered to have a history of safe use, cannot be defined as nutritionally safe *per se*, particularly not when prepared or processed in a novel way [139, 140]. A major challenge with respect to this issue is that most traditional foods have never been subjected to a systematic toxicological or nutritional assessment, especially not in third countries.

As mentioned above, the concept of substantial equivalence aims to identify similarities and differences between the novel food and the conventional comparator with a history of safe use. Expected genotypic and phenotypic alterations as a result

of a genetic modification, e.g. genetic engineering can be determined by targeted analytical approaches. A more challenging goal is the detection of effects not intended by genetic modification. Initially, strategies for the detection of unintended effects, potentially having adverse effects on the human health and / or the environment, were focused on genetically modified crops [7, 134]. However, this might be useful not only for GM-derived foods but also for foods derived from traditional breeding strategies, e.g. crossing and mutation breeding, since the occurrence of unintended effects is not a phenomenon specific to genetic engineering. An overview on the strategies for the detection of intended and unintended effects is shown in Figure 6.

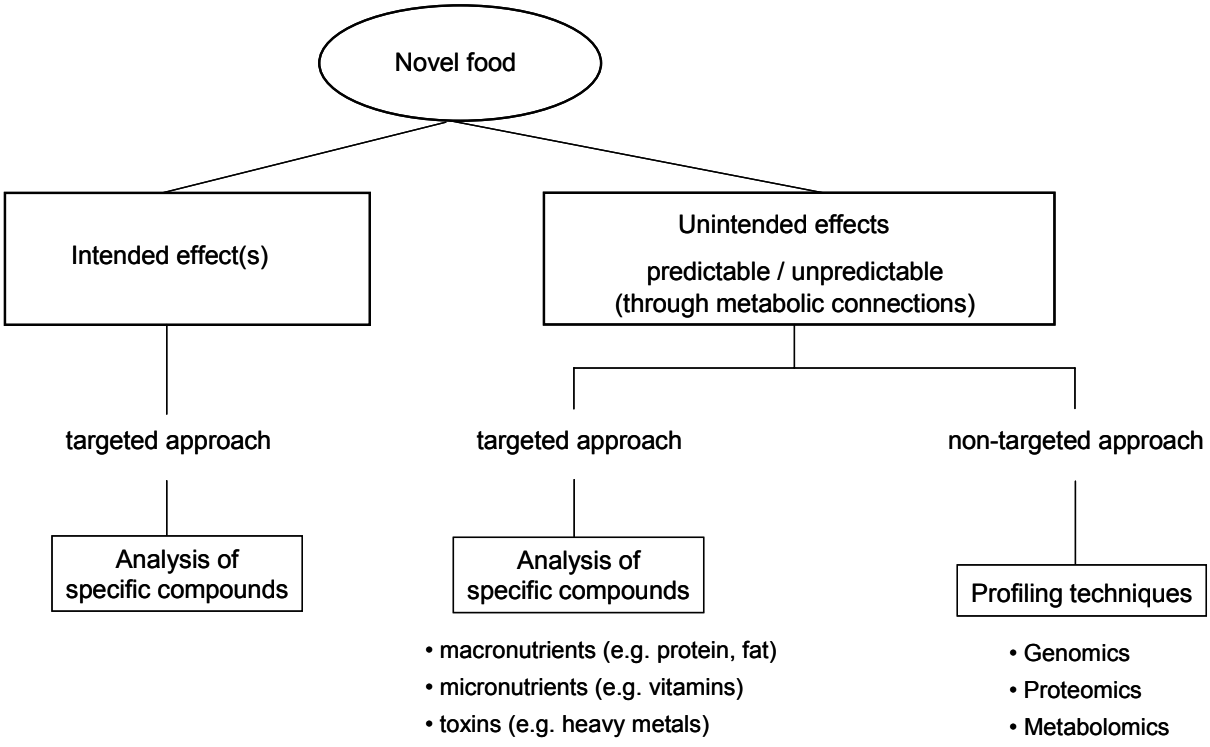

Figure 6: Targeted and non-targeted safety assessment of intended and unintended effects in novel foods

Unintended effects can be subdivided into predictable und unpredictable unintended effects. Predictable unintended effects go beyond the primary induced and expected effect. However, those effects might be explainable through the current knowledge of plant biology and metabolic pathways. Unpredictable unintended effects are differences between modified and parental plant system, which cannot be explained by the present knowledge.

For the detection of potential unintended effects in novel foods, targeted and non-targeted approaches are being discussed [7]. On the food composition level, targeted methods are used for the analysis of specific compounds, e.g. macronutrients, micronutrients and toxins (Figure 6). A comparison of glufosinate-tolerant rice and the non-transgenic counterpart, based on a targeted analysis of key nutrients in rice, revealed compositional equivalence [141]. However, those targeted approaches have limitations owing to to their biased character. That means, only known compounds can be analyzed with respect to these methods. Therefore, unbiased metabolite profiling is considered as additional tool for the safety assessment of GM-derived crops.

2.3.3.2 Functional Metabolite Profiling

In addition to its use in the safety assessment procedures, metabolite profiling is also considered as an approach extending and enhancing the power of functional genomics. Functional genomic approaches have been applied in the analysis of quality traits of major cereals for their further improvement and the development of new cultivars [142]. Metabolite profiling has been suggested to provide valuable data for breeding-driven metabolic engineering of nutritionally important metabolites in crops [8, 9]. During the developmental stages of crops, profound biochemical changes occur. Understanding these metabolic interactions may help to improve the agronomical and nutritional characteristics of the crops [143, 144]. Metabolite profiling has been applied in order to improve the productivity and nutritional quality of tomato fruits [145]. Metabolite profiling techniques have also been applied in plant biodiversity analysis including comparative investigations of breeding systems (e.g. conventional vs. GM), farming practices (e.g. conventional vs. organic) and environmental impacts (e.g. different growing locations / seasons) on crops [12, 13].
Within the functional genomics approach, metabolite profiling may also be used as additional tool for the investigation and characterization of unknown mutation events, e.g. induced through mutation breeding and therefore may help to assist in the elucidation of such events for the discovery of the gene underlying the mutant phenotype. Figure 7 illustrates the link between altered gene sequences in transgenic or mutated plants, effects on the regulation of metabolic levels and potential conclusions which can be made with respect to the mutation event.

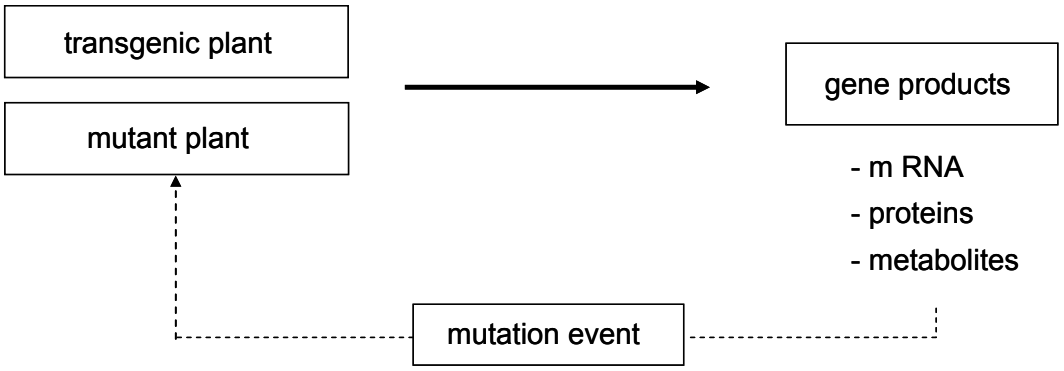

Figure 7: Link between induced genetic modifications and corresponding responses of gene products.

The unbiased and non-targeted screening of a broad spectrum of metabolites by metabolite profiling also increases the probability to detect effects not intended by the mutation process and thus may contribute to a nutritional quality and safety assessment of the induced mutants (see 2.3.3.1).

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

The following chemicals were used:

Acetone	VWR International, Darmstadt
Acetonitril (LiChrosolv®, gradient grade)	Merck, Darmstadt
Argon (purity: 4.6)	Air Liquide, Maisach
4-Chloro-L-phenylalanine (puriss.)	Fluka, Taufkirchen
5α-Cholestane-3β-ol	Fluka, Taufkirchen
Dichloromethane (puriss.)	Fluka, Taufkirchen
Ethanol	Merck, Darmstadt
Formic acid	Fluka, Taufkirchen
Helium (purity: 5.0)	Air Liquide, Maisach
Hexadecane (puriss. p.a.)	Fluka, Taufkirchen
Hexan (p.a.)	VWR International, Darmstadt
Hydrogen (purity: 5.0)	Air Liquide, Maisach
Hydroxylammoniumchloride (for Synthesis)	Merck, Darmstadt
Methanol (LiChrosolv®, gradient grade)	Merck, Darmstadt
Methyl-*tert*-butylether (Driveron S)	Oxeno Olefinchemie, Marl
N-Methyl-*N*-trimethylsilyl-trifluoroacetamide	Fluka, Taufkirchen
N-Trimethylsilylimidazole	Fluka, Taufkirchen
Natriummethoxide Solution (30% in Methanol)	Fluka, Taufkirchen
Natriumsulfat (puriss. p.a.)	VWR International, Darmstadt
Octatriacontane (puriss. p.a.)	Fluka, Taufkirchen
Petroleum ether	Merck, Darmstadt
Phenyl-β-D-glucopyranoside	Fluka, Taufkirchen
Phytic acid dodecasodium salt hydrate	Sigma-Aldrich, Steinheim
Pyridine (puriss. p.a.)	Fluka, Taufkirchen
Hydrochloric acid, 37%	Fluka, Taufkirchen
Nitrogen (purity: 5.0)	Air Liquide, Maisach

Sulfuric acid	Fluka, Taufkirchen
Tetrabutylammoniumhydroxide (puriss. p.a.)	Fluka, Taufkirchen
Tetracosane (puriss. p.a.)	Fluka, Taufkirchen
Toluol	VWR International, Darmstadt
Triacontane (puriss. p.a.)	Fluka, Taufkirchen
Undecane	Fluka, Taufkirchen

Methyl-*tert*-butylether was distilled before usage. All other reagents and solvents were of analytical grade. Authentic reference compounds used for identification and quantification were obtained from Fluka, Merck, Sigma-Aldrich, VWR International and Supelco.

3.1.2 Plant materials

3.1.2.1 Rice

Wild-type rice, low phytic acid mutants and conventional chinese rice samples were obtained from Prof. Dr. Qing-Yao Shu, Zhejiang University, China.

Low phytic acid (lpa) rice

The low phytic acid (*lpa*) rice mutant *Os-lpa*-XQZ-1 and the two *lpa* mutants *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 were grown side by side together with their corresponding *indica* wild-type Xieqingzao B (XQZ) and *japonica* wild-type Xiushui 110 (XS110) during the growing seasons 2003 / 2004 and 2005 / 2006 at field trials in Hangzhou (Zhejiang Academy of Agricultural Sciences (ZAAS), Zhejiang University (ZJU)), Jiaxing, Guangzhou, Hainan and Fuzhou (Zhejiang University (ZJU)), China.

Colored rice

Characteristics of the glutinous and non-glutinous, red and black rice samples are shown in Table 1.

Table 1: Characteristics of colored rice samples

sample code	name	variety	growing place	growing season	color	property
CR1	-[1]	*indica*	Hangzhou	Oct. 2004	red	non-glutinous
CR2	Zhu I	*indica*	Hainan	Apr. 2005	red	non-glutinous
CR3	Xia II	*indica*	Hainan	Apr. 2005	red	non-glutinous
CR4	Honghuami	*indica*	Hainan	Apr. 2005	red	non-glutinous
CR5	Yaxuenuo	*indica*	Hainan	Apr. 2005	black	glutinous
CR6	SB48	*indica*	Hainan	Apr. 2005	black	glutinous
CR7	Zhongxiangnuo	*japonica*	Hainan	Apr. 2005	black	glutinous
CR8	BW8	*indica*	Hainan	Apr. 2005	black	glutinous
CR9	Jiaxingheijing	*japonica*	Hainan	Apr. 2005	black	non-glutinous
CR10	HA1	*japonica*	Hainan	Apr. 2005	black	non-glutinous
CR11	-[1]	*japonica*	Hangzhou	Oct. 2004	black	non-glutinous
CR12	-[1]	*japonica*	Hangzhou	Oct. 2004	black	non-glutinous
CR13	FHW	*japonica*	Hangzhou	Oct. 2004	black	non-glutinous
CR14	Heixiang	*indica*	Hangzhou	Oct. 2004	black	non-glutinous
CR15	-[1]	*japonica*	Hangzhou	Oct. 2004	black	non-glutinous

[1] rice obtained from local Chinese markets

Conventional Chinese rice

The conventional Chinese rice samples were grown from November 2003 to April 2004 at Hainan, China (Table 2). The US and European rice samples were provided by Euryza (Hamburg, Germany) and Primavera (Sulzberg, Germany) (Table 2).

Giant embryo rice

Giant embryo rice mutants II32B-ge, MH-ge2 and MH-ge3 were generated through ^{60}Co γ-irradiation of the hybrid rice maintainer II32B and the rice cultivar Minghui 86, respectively. The giant embryo rice samples were grown during the winter season 2005 at Hainan, China.

Commercial rice

The commercially available brown rice samples (Table 3) were obtained from Euryza (Hamburg, Germany) and Primavera (Mühldorf, Germany).

Table 2: Conventional long grain (LG) and short grain (SG) rice samples

no.	sample code	variety	grain characteristics	no.	sample code	variety	grain characteristics
1	NP1	Indica	LG	27	NO1	Japonica	SG
2	NP5	Indica	LG	28	NO2	Japonica	SG
3	NP8	Indica	LG	29	NO3	Japonica	SG
4	NP9	Indica	LG	30	NO4	Japonica	SG
5	NP10	Indica	LG	31	NO5	Japonica	SG
6	NP14	Indica	LG	32	NO6	Japonica	SG
7	NP16	Indica	LG	33	NO7	Japonica	SG
8	NP17	Indica	LG	34	NO9	Japonica	SG
9	NP18	Indica	LG	35	SC7	Japonica	SG
10	NP19	Indica	LG	36	SC8	Japonica	SG
11	NP22	Indica	LG	37	SC12	Japonica	SG
12	NP24	Indica	LG	38	SC14	Japonica	SG
13	NP26	Indica	LG	39	SC15	Japonica	SG
14	NP28	Indica	LG	40	SC17	Japonica	SG
15	NP29	Indica	LG	41	NF3	Japonica	SG
16	NP30	Indica	LG	42	NF9	Japonica	SG
17	NP31	Indica	LG	43	NF17	Japonica	SG
18	NP33	Indica	LG	44	NF27	Japonica	SG
19	NP36	Indica	LG	45	NF31	Japonica	SG
20	NP42	Indica	LG	46	NF37	Japonica	SG
21	NP43	Indica	LG	47	NF44	Japonica	SG
22	NP46	Indica	LG	48	NF128	Japonica	SG
23	Sb30	Indica	LG	49	NF153	Japonica	SG
24	Sb31	Indica	LG				
25	Sb32	Indica	LG				
26	Sb41	Indica	LG				

Table 3: Commercial brown rice samples

no.	sample code	grain characteristics	growing place	distributor
1	-	n.i.	USA	Euryza
2	-	n.i.	India	Euryza
3	-	LG	Spain	Euryza
4	-	SG	Italy	Euryza
5	Arlette und Ariete	LG	Italy	Primavera
6	Augusto	LG	Italy	Primavera
7	Selenio	SG	Italy	Primavera
8	Masso, Guardiamar	SG	Spain	Primavera

n.i.: no information

3.1.2.2 Soybean

Soybean wild-types and low phytic acid (*lpa*) mutants were obtained from Prof. Dr. Qing-Yao Shu, Zhejiang University, China. *Lpa* mutant lines *Gm-lpa*-TW75-1 and

Gm-lpa-ZC-2 were generated through γ-irradiation of the wild-types Taiwan 75 and Zhechun no. 3 at the Irradiation Center of Zhejiang University, China. Soybean wild-types and corresponding *lpa* mutants were grown side by side within the growing seasons of 2004/2005 to 2005/2006 in field trials at two locations in Hainan and Hangzhou, China.

3.1.2.3 Barley

Grains of malting barley (cultivar Maltasia) were obtained from Lehrstuhl für Technologie der Brauerei I, Technische Universität München, Freising-Weihenstephan, Germany.

3.1.3 Equipment

The following equipments were used:

ACTEVap® Evaporator	Advanced Chemtech, Gießen
Analytical balance Sartorius research R300S	Sartorius, Göttingen
Cyclon mill Cyclotex 1093 (0.5 mm sieve)	Foxx Tecator, Hamburg
Freeze-drying apparatus Christ 1-4 LSC	Martin Christ, Osterode
Glass syringes (10 µL, 50 µL, 250µL)	BGB Analytik,
Silica gel cartridges LiChrolut® Si 60, 3 mL	VWR International, Darmstadt
Manifold Visiprep™	Supelco, München
Membrane filter Spartan 0.45 µm	Schleicher & Schuell, Dassel
Oil bath, consisting of :	
- Heatable magnetic stirrer RH basic	IKA Labortechnik, Staufen
- Thermo stable fluid Labothermol® S	neoLab, Heidelberg
- Glass dish	Zefa Laborservice, Harhausen
- Thermometer ETS-D4	IKA Labortechnik, Staufen
- Magnetic stirring staff	VWR International, Darmstadt
Vacuum rotary evaporator VV2001	Heidolph, Schwabach
Supersonic bath Sonorex RK 100H	Bandelin Electronic, Berlin
Vortexer VF2	IKA Labortechnik, Staufen
Water treatment apparatus Milli-Q plus	Millipore, Eschborn

3.2 METHODS

3.2.1 Analysis of inositol phosphates

3.2.1.1 Preparation of mobile phase

The HPLC mobile phase was prepared by mixing a solution of 485 mL acetonitrile, 515 mL 35 mM formic acid and 10 mL tetrabutylammonium hydroxide. The pH of the mobile phase was adjusted to 4.3 with 75 % sulphuric acid.

3.2.1.2 Extraction procedure

Half a gram of freeze-dried rice flour was defatted in 3 mL petroleum benzene overnight. Petroleum benzene was carefully removed by means of a pipette and remaining solvent was eliminated by a gentle stream of air. Defatted rice flour was extracted under mechanical agitation with 10 mL 2.4 % hydrochloric acid for two hours at room temperature. After centrifugation (15 min, 25 000 g (= 15 000 rpm), 15 °C) 5 ml of the supernatant were diluted with 20 mL deionized water. A silica-based quaternary ammonium anion exchanger column (Chromafix SB (M), 460 mg packing, Macherey-Nagel, Germany) connected to a vacuum manifold (Supelco, Germany) was conditioned with 5 mL deionized water (1 mL/min). After application of the diluted sample extract (1 mL/min) the column was washed with 10 ml deionized water and 10 mL 5 mM hydrochloric acid (1 mL/min). Inositol phosphates were eluted with 5 mL 2 M hydrochloric acid (1 mL/min) into a 10 ml graduated flask which was filled up with deionized water. 2 mL of the purified extract were evaporated under vacuum to dryness by application of an ACTEVap evaporator (Advanced Chemtech, UK). The residue was redissolved in 250 µL HPLC mobile phase. Prior to HPLC analysis the sample was passed through a membrane filter (SPARTAN 13/0.45 RC, Whatman, Germany).

3.2.1.3 HPLC-RI

Separation of inositol phosphates was performed on a macroporous polymer HPLC column PRP-1 (5 µm particle size, 150 x 4.6 mm; Hamilton, Switzerland) at a flow rate of 1 mL/min. The column was kept at 30°C. The HPLC instrument (Kontron, Germany) was equipped with a refractive index detector ERC-7512 (ERMA CR., Japan).

3.2.1.4 Quantification of inositol phosphates

Quantification of phytic acid was based on external calibration using standard solutions of phytic acid dodecasodium salt in the mobile phase (0.25-8.00 mg/mL). Contents of lower inositol phosphates ($InsP_5$, $InsP_4$, $InsP_3$) were quantified on the basis of the calibration curve of phytate considering correction factors for lower inositol phosphates. These were 1.1 for $InsP_5$, 1.5 for $InsP_4$ and 2.4 for $InsP_3$ [108].

3.2.2 Determination of divalent cations

The determination of divalent cations in rice and soybean was carried out at the Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit in Oberschleißheim, Germany. 400 mg of freeze-dried flour was digested with 4 ml nitric acid in a pressure digestion system (Berghof, Germany). The solution was diluted with millipore water to a final volume of 20 ml and analyzed for cadmium by graphite furnace atomic absorption spectrometry using platform atomization with palladium and magnesium nitrate matrix modifiers. Deuterium background correction was used. Contents of calcium, iron and zinc were determined by a simultaneous inductively coupled plasma – optical emission spectrometer with radial view plasma and a cross-flow nebulizer. Scandium (CertiPUR, Merck, Darmstadt) was used as internal standard.

3.2.3 Metabolite profiling of rice

3.2.3.1 Preparation of standard solutions

Internal standard solution for fraction I was prepared by adding n-hexane solutions of undecane (1.5 mL, 2 mg/mL), hexadecane (2.5 mL, 1.5 mg/mL), tetracosane (4 mL, 1.5 mg/mL) and triacontane (4 mL, 1.5 mg/mL) to 15 mg of octatriacontane.
Internal standard solution for fraction II was prepared by bringing 1 mL of a stock solution of 5α-chlolestane-3β-ol in dichloromethane (3 mg/mL) to a final volume of 10 mL with dichloromethane.
Internal standard solution for fraction III was obtained by dissolving 20 mg of phenyl-β-D-glucopyranoside in 25 mL of distilled water.
Internal standard solution for fraction IV was prepared by dissolving 15 mg of p-chloro-L-phenylalanine in 50 µL of distilled water.
Retention time standard mix 1 was identical to the internal standard solution for fraction I
Retention time standard mix 2 was prepared by adding 1.5 mL of n-hexane and n-hexane solutions of hexadecane (2.5 mL, 1.5 mg/mL), tetracosane (4 mL, 1.5 mg/mL) and triacontane (4 mL, 1.5 mg/mL) to 15 mg of octatriacontane.

3.2.3.2 Rough rice

Rough rice grains were manually dehulled by means of a wooden rice dehuller and ground with a cyclone mill equipped with a 500 µm sieve. Before milling, the brown rice was immediately frozen with liquid nitrogen. The milled rice flour was freeze-dried for 48 h and stored at -18 °C.

3.2.3.3 Germination of rice

Brown rice seeds were soaked in tap water (30 °C) for 24 h. After the soaking period, the seeds were placed on a moist filter paper in Petri dishes and incubated for 72 h at 30 °C. Samples were taken after 0 h (brown rice before soaking), 24 h (soaked brown rice), 48 h, 72 h and 96 h. Germinated brown rice was immediately frozen in liquid nitrogen and freeze-dried for 48 hours.

3.2.3.4 Extraction procedure

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

3.2.3.5 Fractionation and analysis of lipids

Fifty microliters of internal standard solution I and 150 µL of internal standard solution II were added to 4 mL of the lipid extract. After evaporation under vacuum to dryness by means of an ACTEVap evaporator (40 °C), the residue was redissolved in 500 µL of methyl *tert*-butyl ether (MTBE), 300 µL of dry methanol, and 50 µL of sodium methylate (5.4 M in methanol). After reaction for 90 min at room temperature, 1 mL of dichloromethane and 2 mL of aqueous 0.35 M hydrochloric acid were added, and the mixture was vigorously shaken. The upper phase was discarded, and the lower phase containing the transmethylated lipids was evaporated to dryness under vacuum (50 °C).

A small amount of anhydrous sodium sulfate was placed on top of a 500 mg silica gel SPE cartridge conditioned with 2.5 mL of *n*-hexane. Transmethylated lipids were redissolved in 250 µL of dichloromethane and transformed to the SPE cartridge. Fraction I was eluted with *n*-hexane / MTBE (100 + 2, v/v, 2 x 3 mL), Fraction II was eluted with *n*-hexane / MTBE (70 + 30, v/v, 2 x 3 mL). After addition of 50 µL of retention time standard 1 to fraction II, both fractions were evaporated to dryness under vacuum (40 °C).

The residue of fraction I was redissolved in 300 µL of dichloromethane, and 1 µL was injected into the gas chromatograph. After the flask had been flushed with argon, the residue of fraction II was redissolved in 250 µL of dry pyridine and silylated with 50 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide in an oil bath (15 min at 70 °C). One microliter of the sample was analyzed by gas chromatography.

3.2.3.6 Fractionation and analysis of polar extract

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

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

3.2.4 Metabolite profiling of barley

3.2.4.1 Preparation of standard solutions

Retention time standard mix 1 and 2 and the internal standard solution for fraction I were prepared as described for the standard solutions in 3.2.3.1.

Internal standard solution for fraction II was obtained by dissolving 15 mg of 5α-chlolestane-3β-ol in 25 mL of dichloromethane.

Internal standard solution for fraction III was obtained by dissolving 40 mg of phenyl-β-D-glucopyranoside in 25 mL of distilled water.
Internal standard solution for fraction IV was prepared by dissolving 20 mg of *p*-chloro-L-phenylalanine in 25 µL of distilled water.

3.2.4.2 Malting procedure

In order to obtain malted barley at different stages of germination, barley grains (cultivar Maltasia) were subjected to a micro-malting procedure, carried out at Institute of Brewing Technolgy I, Technische Universität München, Germany. Initially, barley grains were soaked for 5 hours in tap water (15 °C) until a water content of 30 % was reached. After an incubation stage (5 – 24 h, 15 °C), a further soaking period (24 – 28 h) was conducted to increase the water content to > 38 %. Subsequently, barley grains were incubated between 28 to 144 hours. During incubation, water content in the barley grains increased to 45 % (48 – 72 h). After a total incubation time of 144 h, germinated grains were transferred into a kiln oven (16 h at 50 °C, 1 h at 60 °C, 1 h at 70 °C, 10 h at 80 °C). Samples were taken 5 hours after the start of the micro-malting process and then daily until 144 hours. An additional sample was taken after kilning.

3.2.4.3 Sample preparation

Germinated barley grains were frozen in liquid nitrogen and freeze-dried for 48 hours. Barley grains, germinated barley and malt (kilned germinated barley) were milled and again freeze-dried for 48 hours. The milled flours were stored at -18 °C until analysis.

3.2.4.4 Extraction, fractionation and GC analysis

Extraction of low molecular weight compounds from freeze-dried barley flour was performed as described for rice in 3.2.3.4. In contrast to rice, 200 mg of barley flour was weighted into the cartridge and the flour was soaked in 100 µL of methanol. Fractionation of the lipid extract was performed as described in 3.2.3.5. Instead of 50 µL of internal standard solution 1 (IS 1) and 150 µL of internal standard solution 2

(IS 2), 100 µL IS 1 and 100 µL IS 2 were added to the non-polar extract. Before silylation of fraction II, 100 µL of retention time standard mix 1 was added.
After the addition of internal standard solutions III (150 µL) and IV (150 µL), 2 mL of the polar extract was evaporated to dryness under vacuum (50 °C). The flask was flushed with argon, and the residue was redissolved in 200 µL of dry pyridine and silylated with 100 µL of trimethylsilylimidazole in an oil bath (15 min at 70 °C). The sample was diluted with 400 µL of *n*-hexane and 400 µL of deionized water was added for selective hydrolysis of the silylated derivatives of organic acids and amino acids. After shaking and phase separation, 150 µL of the upper phase (fraction III) was mixed with 75 µL of retention time standard 1, and 1 µL was injected into the gas chromatograph. The fractionation of fraction IV was performed as described in 3.2.3.6. Instead of 200 µL of acetonitrile, 250 µL acetonitrile were used in order to re-dissolve the residue before the final silylation and 100 µL retention time standard mix 2 was added.

3.2.5 Metabolite profiling of soybean

3.2.5.1 Standard solutions and sample preparation

The preparation of the standard solutions was performed as described in 3.2.4.1. Soybean seeds were dried for 72 hours at 60 °C, ground and subsequently passed through a 250 µm sieve. The resulting flour was stored at -18 °C.

3.2.5.2 Extraction, fractionation and GC analysis

Extraction of low molecular weight compounds soybean flour was performed as described for rice in 3.2.3.4. In contrast to rice, 200 mg of soybean flour was weighted into the cartridge and the flour was soaked in 200 µL of methanol.
After adding 100 µL of internal standard solution 1 and 100 µL internal standard solution 2 to the non-polar extract, fractionation of the lipids was as described in 3.2.3.5. Before silylation of fraction II, 100 µL of retention time standard mix 1 was added.

Before fractionation of the polar compounds, the polar fraction was passed through a 0.45 µm filter. After the addition of internal standard solutions III (250 µL) and IV (250 µL), 1 mL of the polar extract was evaporated to dryness under vacuum (50 °C). The silylation of fraction III was performed as described in 3.2.3.6. The sample was diluted with 300 µL of *n*-hexane and 300 µL of deionized water was added. After shaking and phase separation, 200 µL of the upper phase (fraction III) was mixed with 50 µL of retention time standard 1, and 1 µL was injected into the gas chromatograph. In order to obtain fraction IV, 4 mL of the filtered polar extract were evaporated to dryness. Silylation and phase separation conditions were identical to 3.2.3.6.

3.2.6 Gas chromatography

Gas chromatography (GC) was performed on a Focus GC (Thermo, Austin, TX) equipped with a flame ionization detector (FID).

GC conditions:

Injection volume:	1 µL
Column:	DB1, J & W Scientific, Folsom, CA 60 m x 0.32 mm i.d. fused silica capillary coated with a 0.25 µm film of polydimethylsiloxane
Injector type:	Split / Splitless
Split flow:	25 mL/min
Carrier gas:	Hydrogen
Flow modus:	1.8 mL, constant flow
Injector temperature:	250 °C
Detector temperature:	320 °C
Temperature program:	100 °C to 320 °C at 4 °C/min, 25 min hold

GC-FID data were acquired using the commercially available software Chrom-Card 2.3 (Thermo Electron). The sampling rate was set to 600 data points/min. Automated peak integration within the retention time range between 5 min and 50 min was performed using a peak width of 5 sec and a peak threshold of 5. For integration of peaks eluting at retention times higher than 50 min, peak threshold was set to 9 and

peak width was adjusted to 15 sec. Peaks exhibiting an area lower than 3000 µV*min were not taken into account.

3.2.7 Gas chromatography – mass spectrometry

Gas chromatography – mass spectrometry (GC-MS) analysis was performed on a Finnigan TraceGC ultra coupled to a quadrupole mass selective detector Finnigan Trace DSQ (Thermo, Austin, TX).

GC conditions:

Injection volume:	1 µL
Column:	FactorFOUR VF-1ms, Varian 60 m x 0.32 mm i.d. fused silica capillary coated with a 0.25 µm film of polydimethylsiloxane
Injector type:	Split / Splitless
Split flow:	15 mL/min
Carrier gas:	Helium
Flow modus:	1.0 mL, constant flow
Injector temperature:	280 °C
Detector temperature:	320 °C
Temperature program:	100 °C to 320 °C at 4 °C/min, 10 min hold
MS conditions:	
Interface temperature:	320 °C
Ionization method:	EI+, 70 eV
Source temperature:	250 °C
Scan range:	40 – 700 Da (full scan mode)
Scan interval:	0.4 s

GC-MS data were acquired using the commercially available software Xcalibur 1.4 SR1 (Thermo, Austin, TX).

3.2.8 Response factor

For the determination of response factors, reference compounds were silylated and analyzed under the conditions given in 3.2.6 and calculated according to Equation 1.

Equation 1: $$R = (\frac{m_A * H_{IS}}{H_A * m_{IS}})$$

The calculated response factors for *myo*-inositol, galactose, galactinol and raffinose were as follows: 0.348, 0.484, 0.668 and 0.764.

3.2.9 Recovery

Recoveries were determined by spiking rice flour with known amounts of reference compounds before extraction process. 50 µl of a solution of galactose (0.4 mg/ml), *myo*-inositol (1.2 mg/ml), raffinose (8 mg/ml) and galactinol (0.5 mg/ml) in deionized water were added to each of three samples of 600 mg of freeze-dried rice flour. The samples were analyzed as described in 3.2.3. In addition, three samples of the same rice flour were analyzed without spiking. The differences in the peak heights of spiked and unspiked samples were compared to those of known amounts of reference standards. The recoveries for the selected compounds were as follows: galactose, 74 %; *myo*-inositol, 58 %; galactinol, 69 %; raffinose, 71 %.

3.2.10 Analysis of metabolite profiling data

3.2.10.1 Identification of rice, barley and soybean constituents

Polar and non-polar low metabolite weight rice, barley and soybean constituents were identified by comparing retention times and mass spectra with those of silylated reference compounds and by comparing mass spectra with the entries of the mass spectral libraries NIST 2002 (National Institute of Standards and Technology, Gaithersburg, Maryland) and Golm Metabolome Database [146].

3.2.10.2 Statistical assessment

Plant samples were analyzed in triplicate. Peak heights and retention times were exported from Chrom-Card 2.3 (Thermo, Austin, TX) to Chrom*pare* version 1.1 [147] for comparative univariate analysis. Multivariate statistical analyses were performed by means of Principal Component Analysis (PCA) and Analysis of Variance (ANOVA). They were conducted using XSSTAT 7.5.2 (Addinsoft, France) and SYSTAT 11 (Systat Software Inc., Richmond, California).

4 RESULTS AND DISCUSSION

4.1 ANALYSIS OF GERMINATING SEEDS

4.1.1 Introduction

During the past years, metabolite profiling has been applied to assess genotypic and phenotypic diversity in plants. Comparative investigation of breeding systems, e.g. conventional vs. genetically modified crops [11], has been conducted. Moreover, the influence of farming practices and environmental impacts on crops have been investigated [13]. Metabolite profiling techniques have also been employed to follow the development of plant systems, e.g. the metabolic changes in Arabidopsis seeds [15] and strawberry fruits [16]. For cereals, metabolomic data have been used to investigate changes during tillering [14], to reveal metabolic modulation in foliage [148], to phenotype natural variants [10], to analyze toxicological effects on plant growth [149] and to investigate the influence of mineral deficiencies on the metabolite profile [150].

Germination of seeds represents an important stage in the development of plants. This phase is characterized by metabolic processes. Distinct and time-dependent alterations in metabolite levels are to be expected and metabolite profiling should be a suitable analytical tool to provide a comprehensive picture of these changes. Therefore, the aim of this study was to apply metabolite profiling based on GC-MS to germinating cereal seeds.

Targeted analyses have demonstrated that the germination of rice and barley is accompanied by a spectrum of significant changes in metabolite contents [25, 30, 32, 151]. Proteomics and transcriptomics-based investigations of rice seeds [152] and barley [153] during germination have also been performed. However, no metabolite profiling - based investigations of the germination process have been described. The objectives of this study were: (i) to analyze a broad spectrum of low molecular weight compounds covering a wide range of chemical classes in the germinating seed materials; (ii) to test the applied GC-MS approach for its suitability to reflect the germination process by a time-dependent clustering based on multivariate analysis;

and, (iii) to quantify constituents and to follow their dynamic changes during the germination.

4.1.2 Germinating rice seeds

Three brown rice samples (II32B-ge, MH-ge2 and MH-ge3) were subjected to germination. The rice seeds were soaked for 24h in tap water and subsequently incubated for 72h at an ambient temperature of 30°C on wet filter paper. The morphological changes of the rice kernels observed upon soaking and incubation are shown in Figure 8. The initial enlargements of the rice embryos and the subsequent formations of shoots and roots are in good agreement with changes described for germinated rice grains [154]. By definition, germination of seeds begins with water uptake (imbibition) by the quiescent seed and ends with the start of elongation by the embryonic axis, usually the radicle [155].

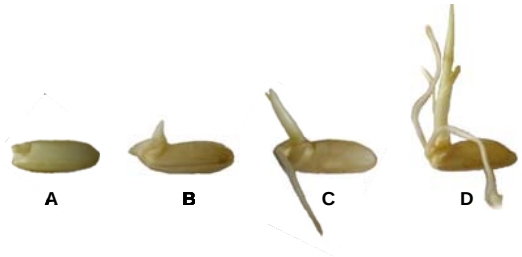

Figure 8: Germinated brown rice after a soaking period of 24h (A) and after incubation times of 48h (B), 72h (C) and 96h (D).

4.1.2.1 Detection and Identification of Metabolites

Samples taken at different time points during the germination process were immediately freeze-dried and ground. The flour was subjected to an extraction and fractionation methodology developed for rice grains [118]. The approach is based on consecutive extraction of lipids and polar compounds (Figure 4) which is comparable to other strategies as regards the extraction of metabolites with solvents differing in polarity and subsequent derivatization [115, 119]. However, additional transesterification-solid phase extraction (lipids) and selective hydrolysis of silylated derivatives (polar compounds) were applied to separate major from minor constituents. This procedure results in four fractions containing fatty acid methyl

esters and hydrocarbons (fraction I), free fatty acids, alcohols and sterols (fraction II), sugars and sugar alcohols (fraction III), acids, amino acids and amines (fraction IV). The four fractions obtained were analyzed by capillary gas chromatography. GC-FID analysis resulted in the detection of a total of 615 distinct peaks in the course of the germination of the brown rice. As examples, the four fractions obtained from the germinated rice II32B-ge are shown in Figure 9. GC-MS analysis enabled the identification of 174 peaks in the germinated rice II32B-ge: 36 saturated / unsaturated fatty acid methyl esters and 16 hydrocarbons in fraction I (Table 4), 21 free fatty acids, 10 fatty alcohols, 14 sterols / triterpenic alcohols, two phenolic compounds and three tocopherols in fraction II (Table 5), 13 sugars and sugar alcohols in fraction III, 22 organic and inorganic acids, 34 amino acids / amines and three further polar compounds in fraction IV (Table 6).

Table 4: Compounds identified in fraction I in germinated rice II32B-ge

no.	compound	ident.[a]	no.	compound	ident.[a]	no.	compound	ident.[a]
saturated FAME[b]			*unsaturated FAME*			*hydrocarbons*		
1	C10:0	A	8	C14:1	A	2	C14	A
3	C11:0	A	12	C15:1	A	4	C15	A
5	C12:0	A	16	C16:1	C	9	C17	A
6	C13:0	A	17	C16:1 cis	A	13	C18	A
10	C14:0	A	21	C17:1	A	18	C19	A
14	C15:0	A	24	C18:1	A	22	C20	A
19	C16:0	A	28	C19:1	B	29	C22	A
23	C17:0	A	32	C20:1	A	33	C23	A
27	C18:0	A	37	C22:1	A	38	C25	A
30	C19:0	A	42	C24:1	A	40	C26	A
34	C20:0	A				43	C27	A
35	C21:0	A	7	C14:2	C	45	C28	A
39	C22:0	A	11	C15:2	C	46	squalene	A
41	C23:0	A	15	C16:2	C	47	cholestane	C
44	C24:0	A	20	C17:2	C	48	C29	A
49	C26:0	A	25	C18:2	A	50	C31	A
51	C28:0	A	31	C20:2	A			
52	C30:0	A	36	C22:2	A			
			26	C18:3	A			

[a] Identification according to

A mass spectrometric data and retention time of reference compound

B mass spectrometric data and retention index of the Golm metabolome database [146]

C NIST 02 MS library

[b] fatty acid methyl esters

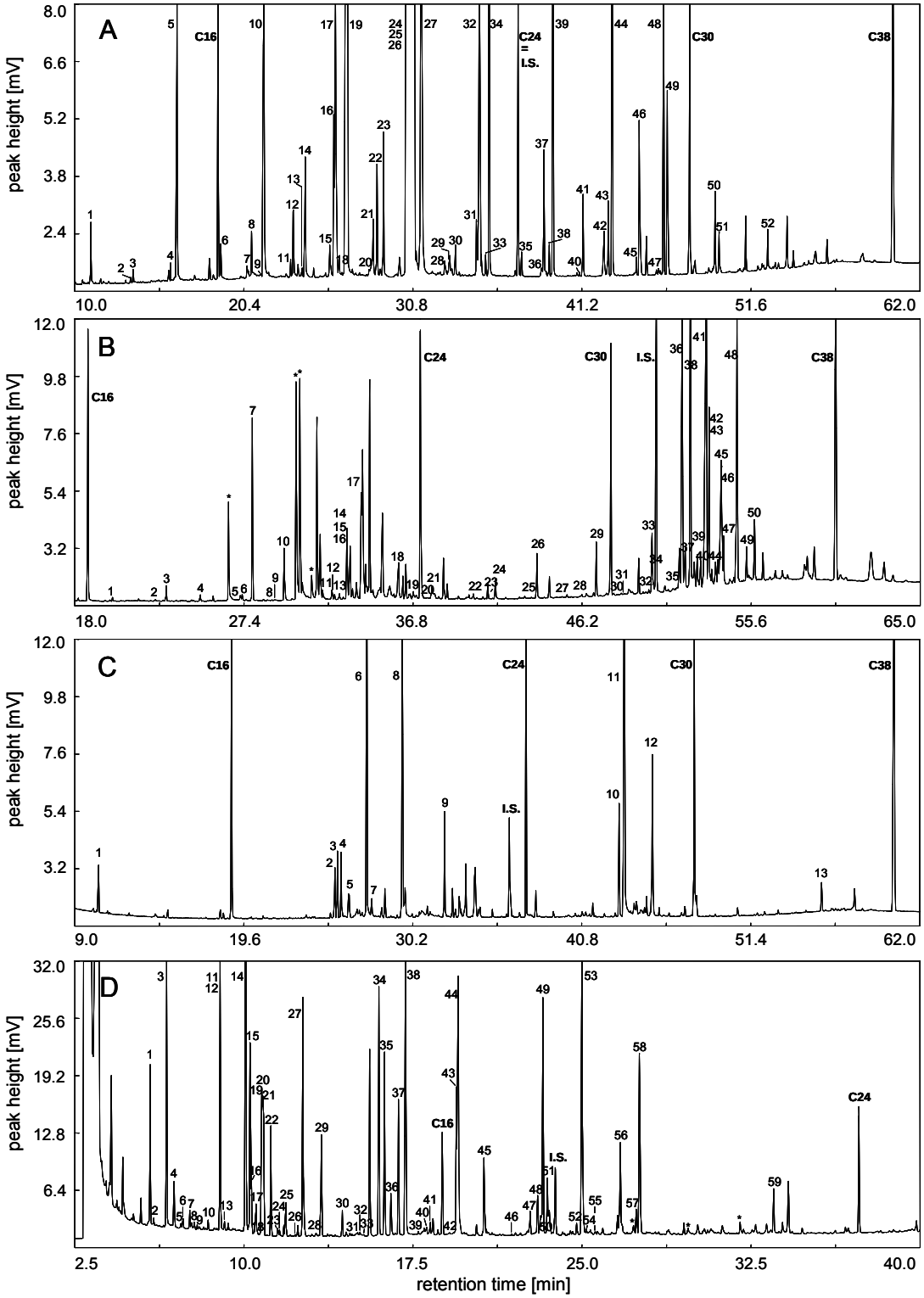

Figure 9: CG-FID chromatograms of fraction I (A), fraction II (B), fraction III (C), and fraction IV (D) obtained from germinated rice II32B-ge. I.S.: internal standards tetradecane (A), 5α-cholestane-3ß-ol (B), phenyl-ß-D-glucopyranoside (C), *p*-chloro-L-phenylalanine (D); C16, C24, C30, C38: retention time standards; Identification of peaks is given in Tables 4 – 6. Residual FAME in fraction II and residual sugars in fraction IV are marked with asterisks.

Table 5: TMS derivatives of compounds identified in fraction II in germinated rice II32B-ge

no.	compound	ident[a]	no.	compound	ident[a]	no.	compound	ident[a]
free fatty acids			*fatty alcohols*			*sterols and triterpenic alcohols*		
1	C12:0	A	7	C16:0	A	34	cholesterol	A
2	C13:0	A	12	C18:0	A	36	campesterol	A
4	C14:0	A	13	phytol	A	37	campestanol	A
6	C15:0	A	19	C20:0	A	38	stigmasterol	A
8	C16:1	C	22	C22:0	A	39	Δ^7-campestenol	E
9	C16:1 (*cis* 9)	A	26	C24:0	C	41	ß-sitosterol	A
10	C16:0	A	29	C26:0	A	42	sitostanol	A
11	C17:0	A	33	C28:0	A	43	Δ^5-avenasterol	A
14	C18:3	A	40	C30:0	D	44	gramisterol	F
15	C18:2	A	50	C32:0	D	45	Δ^7-stigmastenol	F
16	C18:1	A				46	cycloartenol	A
17	C18:0	A	*phenolic compounds*			47	Δ^7-avenasterol	F
18	C19:0	A	3	methyl *p*-hydroxy-		48	24-methylene-	
20	C20:1	A		cinnamate	A		cylcoartanol	A
21	C20:0	A	5	methyl ferulate	A	49	citrostadienol	F
23	C22:1	A						
24	C22:0	A	*tocopherols*					
25	C23:0	A	28	δ-tocopherol	A			
27	C24:0	A	30	γ-tocopherol	B			
31	C26:0	B	32	α-tocopherol	A			
35	C28:0	A						

[a] Identification according to

- A mass spectrometric data and retention time of reference compound
- B mass spectrometric data and retention index of the Golm metabolome database [146]
- C NIST 02 MS library
- D MS data
- E Xu and Godber 1999 [156]
- F Kamal-Eldin 1992 [157]

The number of peaks detected and identified in this study was in the same order of magnitude or even higher than in comparable metabolite profiling studies performed in other plant materials. A GC-MS approach on rice plants during the tillering phase revealed around 300 metabolites of which 155 could be identified using a standards library [14]. Metabolite profiling of tomato plants during leaves and fruit development resulted in the identification of 71 and 82 compounds, respectively [8]. However, due to the used metabolic profiling protocol, only polar metabolites were included into the investigation of tomato leaves and fruit.

Table 6: TMS derivatives of compounds identified in fractions III (sugars and sugar alcohols) and IV (acids, amino acids, amines) in germinated rice II32B-ge

no.	compound	ident[a]	no.	compound	ident[a]
sugars and sugar alcohols			*amino acids and amines*		
1	glycerol	A	3	alanine	A
2,3,4	fructose	A	9	2-aminobutyric acid	A
5,7	galactose	A	10	ß-alanine	A
6,8	glucose	A	11	valine	A
9	*myo*-inositol	A	12	norvaline	A
10,11	sucrose	A	15	leucine	A
12	trehalose	A	16	ethanolamine	A
13	raffinose	A	18	γ-aminobutyric acid	A
			19	isoleucine	A
acids			20	proline	A
1	lactic acid	A	22	glycine	A
2	glycolic acid	A	27	serine	A
5	3-hydroxypropanoic acid	C	29	threonine	A
6	pyruvic acid	A	30	ß-alanine	A
7	ß-hydroxybutyric acid	A	31	homoserine	A
8	3-methyl-2-hydroxybutyric acid	C	33	ß-aminoisobutyric acid	A
13	γ-hydroxybutyric acid	C	35	pyroglutamic acid	A
14	phosphoric acid	A	36	methionine	A
17	maleic acid	A	37	aspartic acid	A
21	succinic acid	A	38	γ-aminobutyric acid	A
24	glyceric acid	A	39	5-hydroxynorvaline	C
25	fumaric acid	A	42	2-aminopimelic acid	C
26	pyrrole-2-carboxylic acid	A	43	glutamic acid	A
28	glutaric acid	A	44	phenylalanine	A
32	2-piperidinecarboxylic acid	C	45	asparagine	A
34	malic acid	A	46	α-aminoadipic acid	A
40	threonic acid	A	48	putrescine	A
41	3-phenyl lactic acid	A	49	glutamine	B
47	*cis*-aconitic acid	C	52	citrulline	A
50	3-glycerophosphoric acid	B	54	ornithine	A
51	2-aminoethylphosphoric acid	C	56	histidine	A
53	citric acid	A	57	lysine	A
			58	tyrosine	A
others			59	tryptophan	A
4	2-pyrrolidinone	A			
23	2,4-hydroxypyrimidine	C			
55	adenine	A			

[a] Identification according to

A mass spectrometric data and retention time of reference compound

B mass spectrometric data and retention index of the Golm metabolome database [146]

C NIST 02 MS library

4.1.2.2 Principal Component Analysis

For the investigation of time-dependent metabolic changes in the course of germination, data obtained by GC-FID analyses of II32B-ge, MH-ge2 and MH-ge3 were statistically analyzed using PCA. For consolidation of the raw data, peak heights and corresponding retention times were exported to Chrom*pare*, a software tool developed for comparative analysis of metabolite profiling data [147]. Chrom*pare* automatically corrects retention time shifts on the basis of retention time standards and standardizes peak heights on the basis of internal standards added before the fractionation process. PCA was performed for each single fraction and additionally for the combined fractions I – IV taking into account a total of 615 distinct peaks obtained from GC-FID metabolite profiling. Scores plots obtained for the single and combined fractions are shown in Figure 10.

The metabolic changes are reflected by time-dependent shifts of the scores for the first two principal components PC1 and PC2. For the non-polar fractions, the first two principal components explained 51 % of the total variation in fraction I (Figure 10A) and 57 % in fraction II (Figure 10B). An even more pronounced variance was covered by the first two principal components for the polar fractions: 65 % in fraction III (Figure 10C) and 72 % in fraction IV (Figure 10D), respectively. In the scores plot based on the combined metabolite profiling data from all four fractions a variance of 52 % was covered by PC1 and PC2 (Figure 10E). Similar coverages of variance have been reported in other metabolite profiling studies [11, 13]. A comparison of wild-type and transgenic potatoes, considering 88 partly unknown metabolites, resulted in a clear clustering of wild-type and transgenic genotypes; 68 % of the total variance was covered by the first two principal components [11]. Recently, a principal component analysis was conducted with 44 metabolites from tomato flesh and seeds at different stages of fruit development. It was shown that 67 % of the total variability was explained by the first two PCs [17].

The three rice samples II32B-ge, MH-ge2 and MH-ge3 showed similar score patterns depending on the time of germination. However, a differentiation between II32B-ge, derived from the hybrid rice maintainer II32B, and the two Minghui 86-derived rice lines MH-ge2 and MH-ge3 was observed.

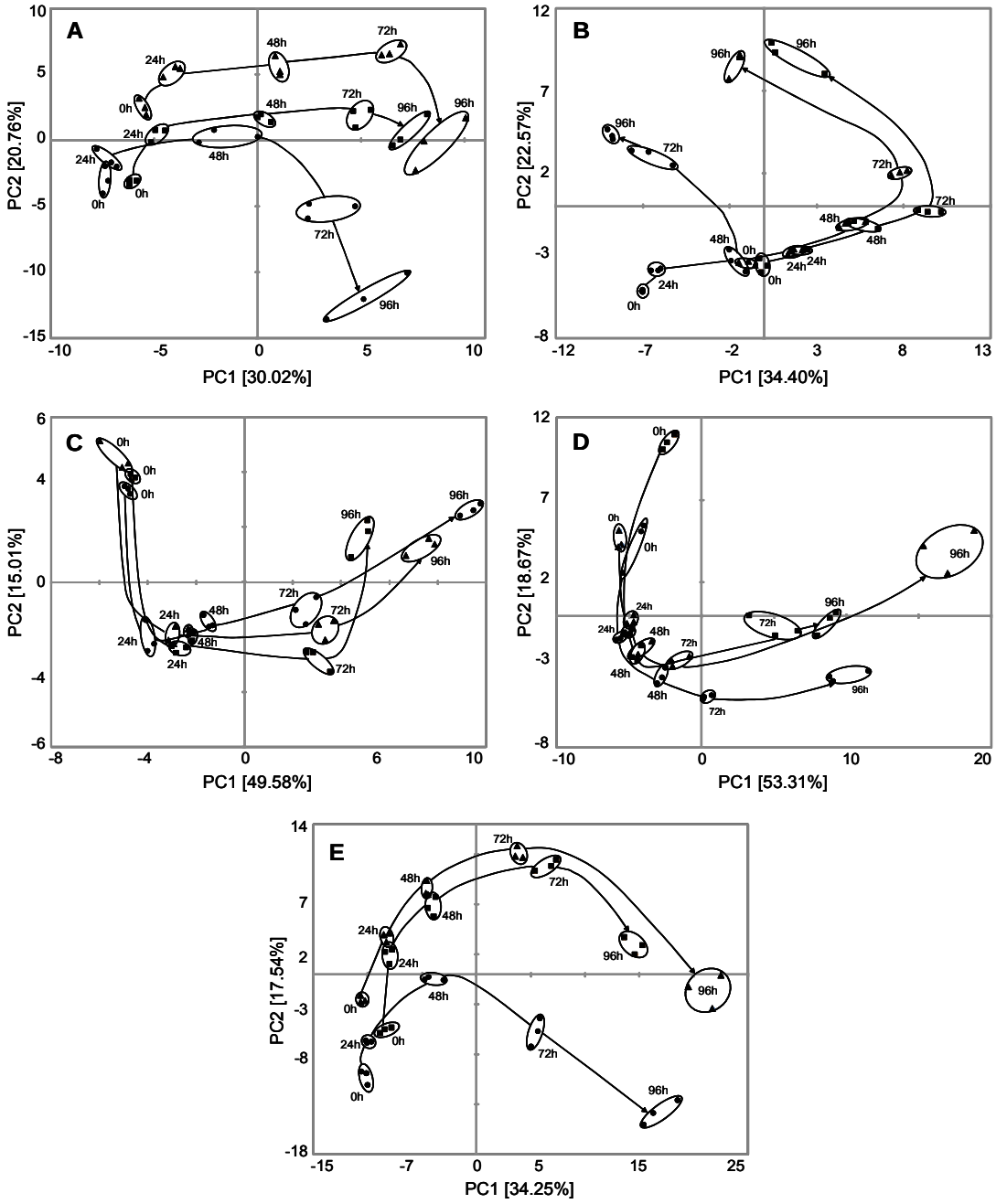

Figure 10: Principal component analysis of standardized GC-FID metabolite profiling data of fractions I (A), II (B), III (C) and IV (D), and of combined fractions I - IV (E) in the course of the germination (0, 24, 48, 72, 96h) of II32B-ge (●), MH-ge2 (▲) and MH-ge3 (■).

In order to identify the metabolites mainly responsible for the variance within the scores plots, the analysis of the corresponding loadings, taking into account the data of all 615 peaks detected in fractions I – IV, has been included in the investigation of rice seed in the course of germination (Figure 11). Polar metabolites were found to

be major contributors to the separation along the first principal component, whereas predominantly non-polar metabolites were responsible for the separation along the second principal component indicating more pronounced changes of polar metabolites during the germination process. The corresponding loading plots for the single fractions II and IV are shown in Figures 12A and 12B, respectively.

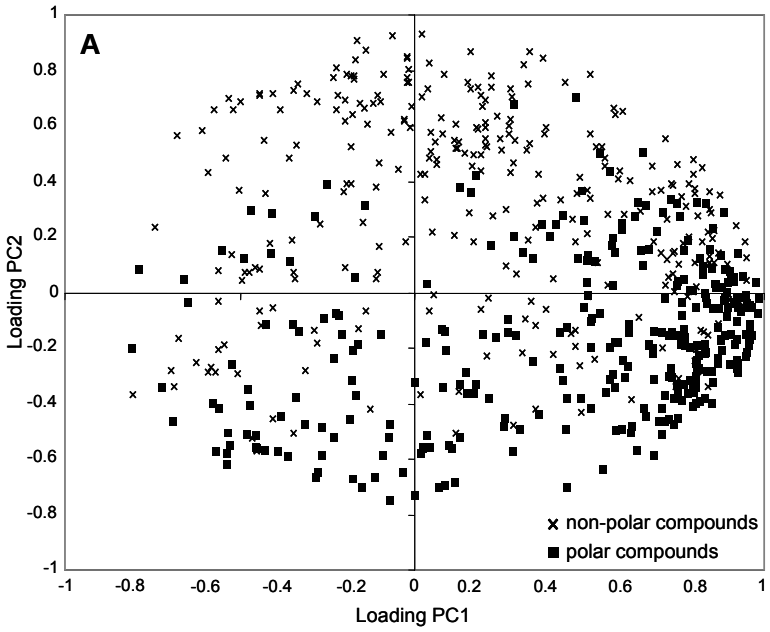

Figure 11: Loading plot of standardized GC-FID metabolite profiling data from non-polar and polar compounds taking into account 615 peaks from fractions I-IV.

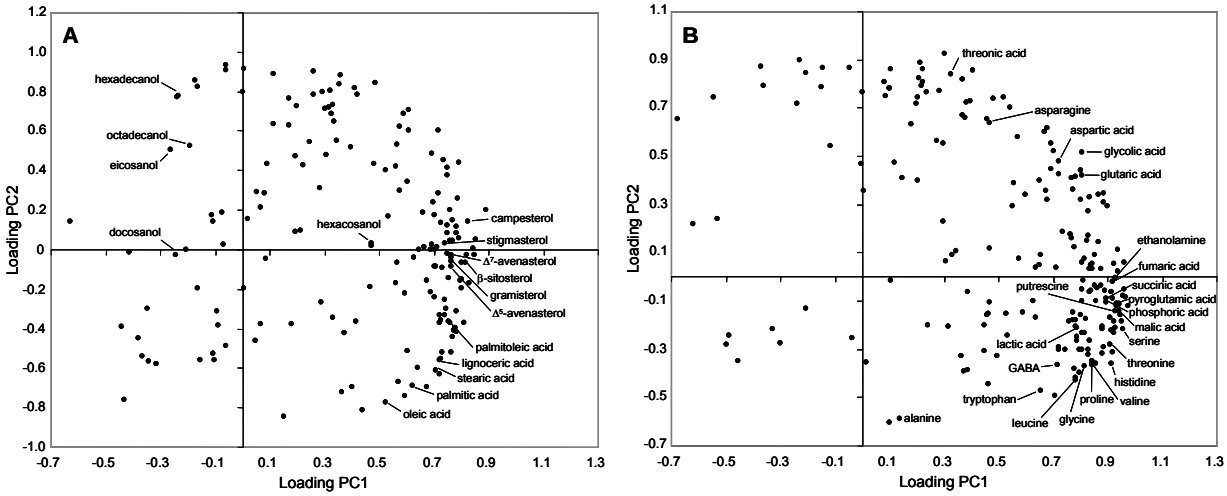

Figure 12: Loading plots of standardized GC-FID metabolite profiling data from fraction II (minor lipids) (A) and fraction IV (acids, amino acids and amines) (B).

4.1.2.3 Relative Quantification of Compounds

Quantitative comparisons were based on standardized peak heights. The sum of standardized peak heights obtained from GC-FID analyses the four fractions I-IV of the ungerminated (0h) and germinated (96h) GE rice mutants II32B-ge, MH-ge2 and MH-ge3 are shown for the lipids in Figure 13 and for the polar compounds in Figure 15. Additionally, changes observed for representatives of different classes of non-polar and polar metabolites in the course of germination are shown in Figures 14 and 16.

The fatty acid methyl esters detected in fraction I result from transesterification of the lipid extract and reflect the total contents and the fatty acid compositions of the rice triglycerides. The methyl esters of palmitic, stearic and arachidic acid were shown to be the major saturated ones in the three ungerminated brown rice samples representing on average 91% of the totally covered saturated FAME.

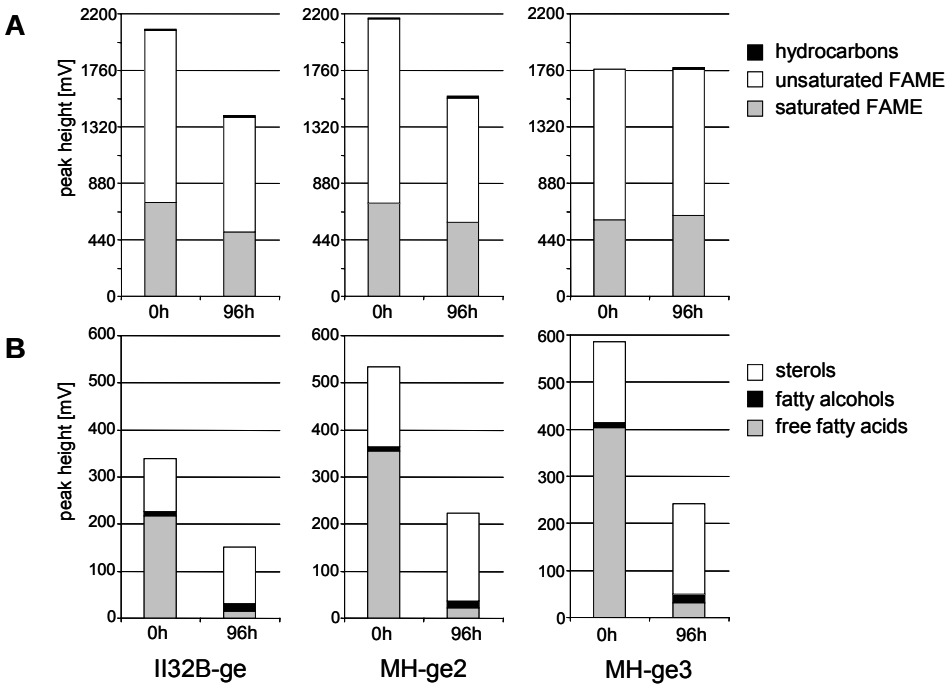

Figure 13: Sum of standardized peak heights and distribution of compound classes in the non-polar fractions I (A) and II (B) obtained from II32B-ge, MH-ge2 and MH-ge3 in ungerminated (0h) and germinated (96h) rice.

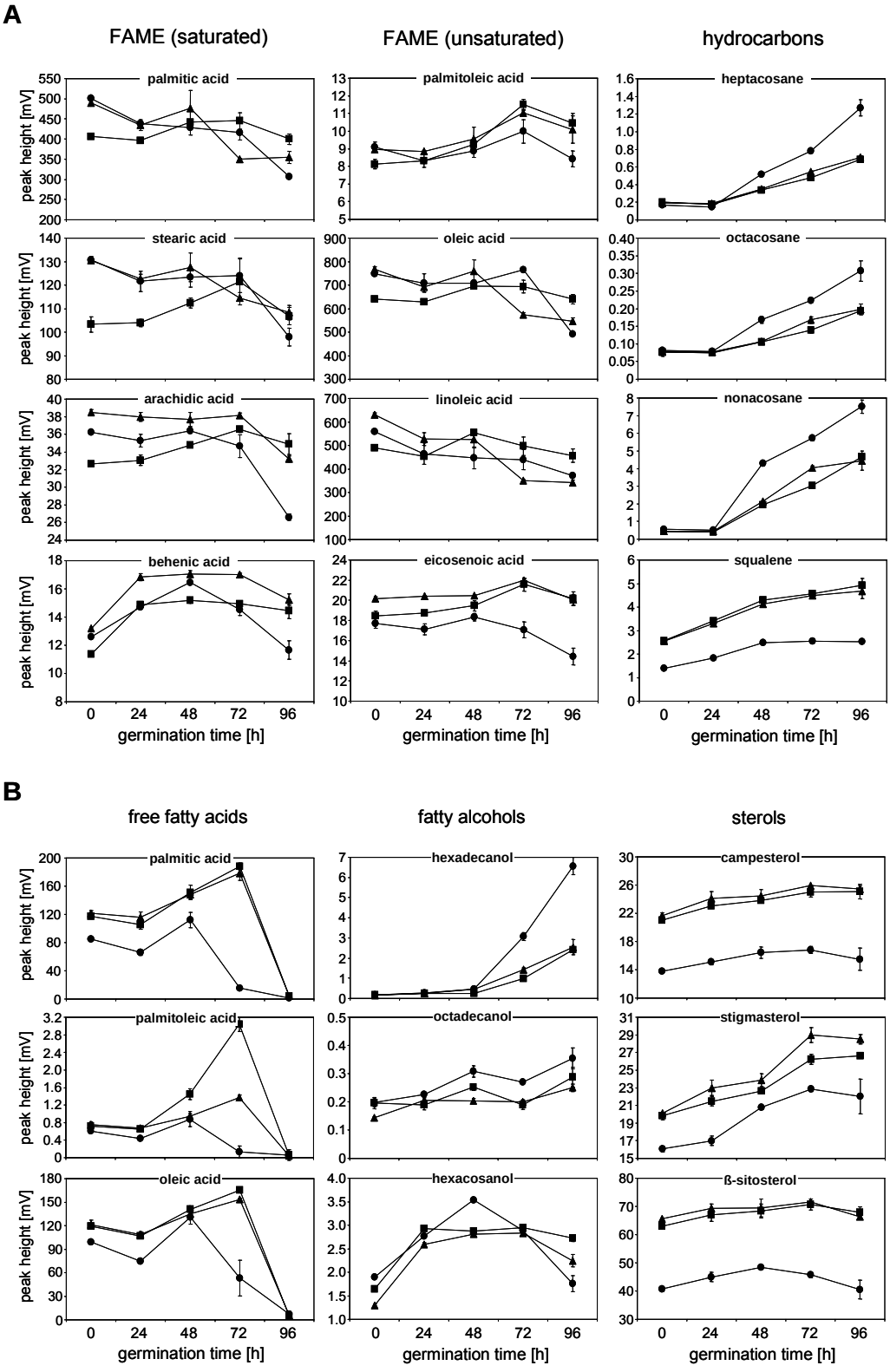

Figure 14: Standardized peak heights for selected fatty acid methyl esters (FAME; saturated and unsaturated) and hydrocarbons obtained from fraction I (A) and free fatty acids, fatty alcohols and sterols obtained from fraction II (B) in the course of the germination of II32B-ge (●), MH-ge2 (▲) and MH-ge3 (■).

Oleic, linoleic and eicosenoic acid are the major unsaturated triglyceride-derived fatty acids in brown rice representing over 99 % of the total unsaturated FAME. After 96h of germination, slightly decreased contents of the total fatty acid methyl esters were observed for II32B-ge (-32 %) and MH-ge2 (-20 %), whereas the level in MH-ge3 remained almost unchanged (Figures 13A and 14A).

Heptacosane, octacosane, nonacosane and squalene were major representatives of the hydrocarbons detected in fraction I. The peak heights determined for these metabolites significantly increased in the course of the germination (Figure 14A). After an incubation for 96h, concentrations of total hydrocarbons were increased by up to +400 % in II32B-ge. Hydrocarbons are known constituents of starch lipids in rice [158]. A total lipid content of 0.7 % in rice starch has been reported [159]. Germination of rice involves cytolytic and amylolytic degradation of rice starch which might result in an improved extraction of grain lipid constituents associated with polysaccharides.

Major free saturated and unsaturated fatty acids in the brown rice samples were palmitic, stearic, oleic, linoleic and linolenic acid representing on average 95% of the total free fatty acids. Compared to brown rice, germinated rice incubated for 96h exhibited significantly decreased contents of free fatty acids (on average: -93 %) (Figure 13B). As shown in Figure 14B for palmitic, palmitoleic and oleic acid, concentrations of free fatty acids increased in the initial stage of the incubation, but decreased rapidly after 72 h. In contrast, contents of fatty alcohols, e.g. hexadecanol, octadecanol and hexacosanol, were shown to be increased in germinated rice compared to ungerminated rice.

The overall changes in sterol levels were relatively small. Germination resulted in slightly increased concentrations in germinated rice ranging from +6 % in II32B-ge to +12 % in MH-ge3, respectively. Changes observed for the three major sterols campesterol, stigmasterol and ß-sitosterol are shown in Figure 14B. Germination of tobacco seeds resulted in changes of the three major sterols similar to those seen in the present study [160].

The changes of total sugar contents were relatively small. The levels of sugars were moderately decreased by -11 % for II32B-ge to -38 % for MH-ge3 (Figure 15A). However, compared to non-polar metabolites, the changes observed for polar constituents were much more pronounced. Levels of monosaccharides (e.g. glucose,

galactose) increased significantly in the course of the germination whereas those of di- and trisaccharides (e.g. sucrose, raffinose) were drastically reduced (Figure 16A).

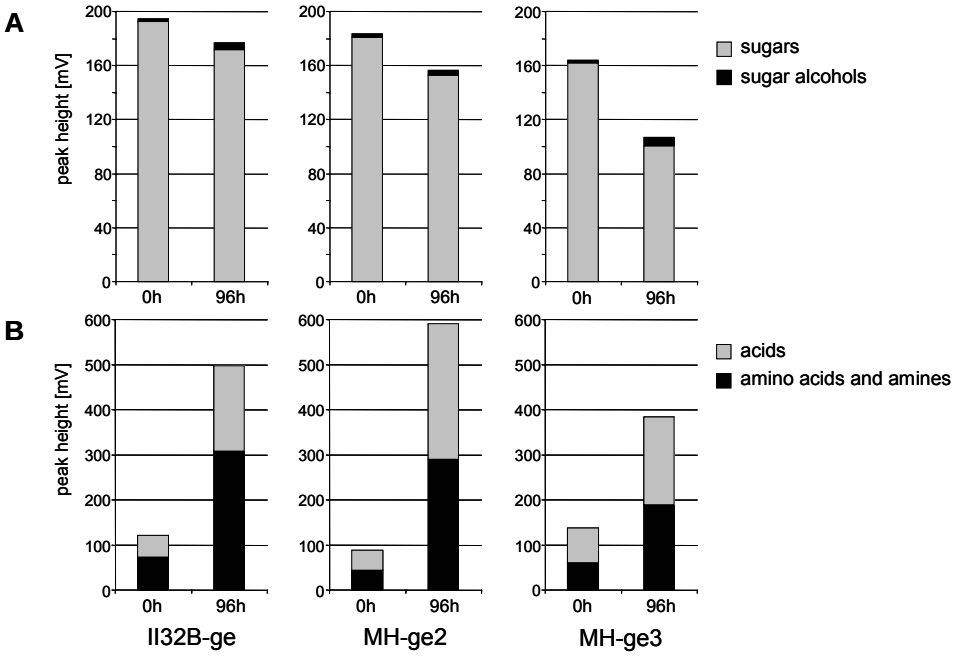

Figure 15: Sum of standardized peak heights and distribution of compound classes in the polar fractions III (A) and IV (B) obtained from II32B-ge, MH-ge2 and MH-ge3 in ungerminated (0h) and germinated (96h) rice.

Analysis of rice starch during a germination period of 7 days revealed a decrease by - 43 %, whereas an up to 40-fold increase in the level of reducing sugars was observed [30]. Similarly to the lipid degradation, starch degradation and accumulation of reducing sugars in germinating rice were shown to be greatly influenced by the incubation conditions [31]. As shown for glycerol and *myo*-inositol, no consistent patterns were observed for sugar alcohols (Figure 16A).

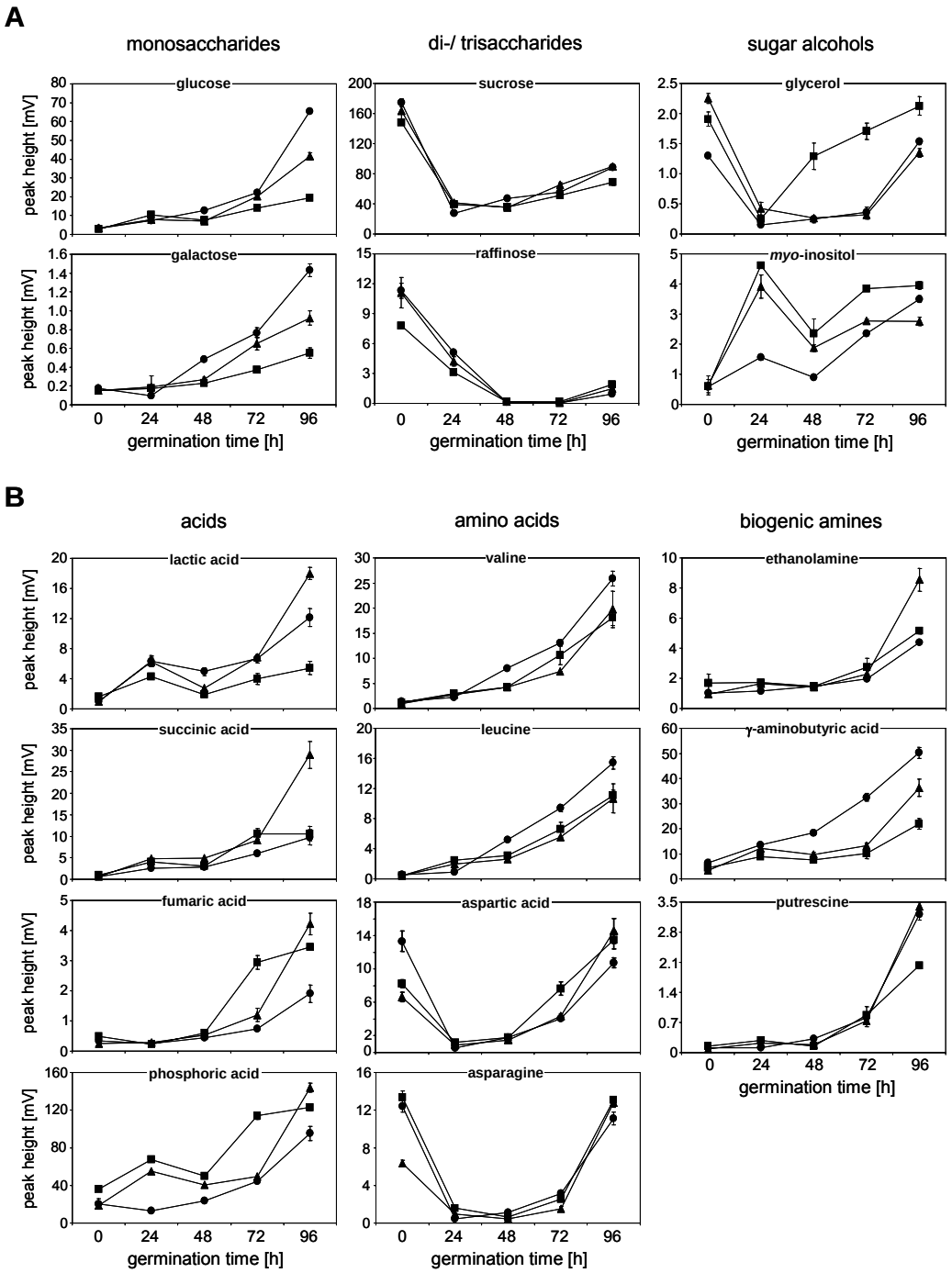

Figure 16: Standardized peak heights for selected sugars and sugar alcohols obtained from fraction III (A) and acids, amino acids and amines obtained from fraction IV (B) in the course of germination of II32B-ge (●), MH-ge2 (▲) and MH-ge3 (■).

Levels of acids, amino acids and amines in fraction IV increased significantly in the course of the germination (Figure 15B). Representative GC-FID chromatograms obtained from fraction IV of II32B-ge during the different stages of the germination process are shown in Figure 17.

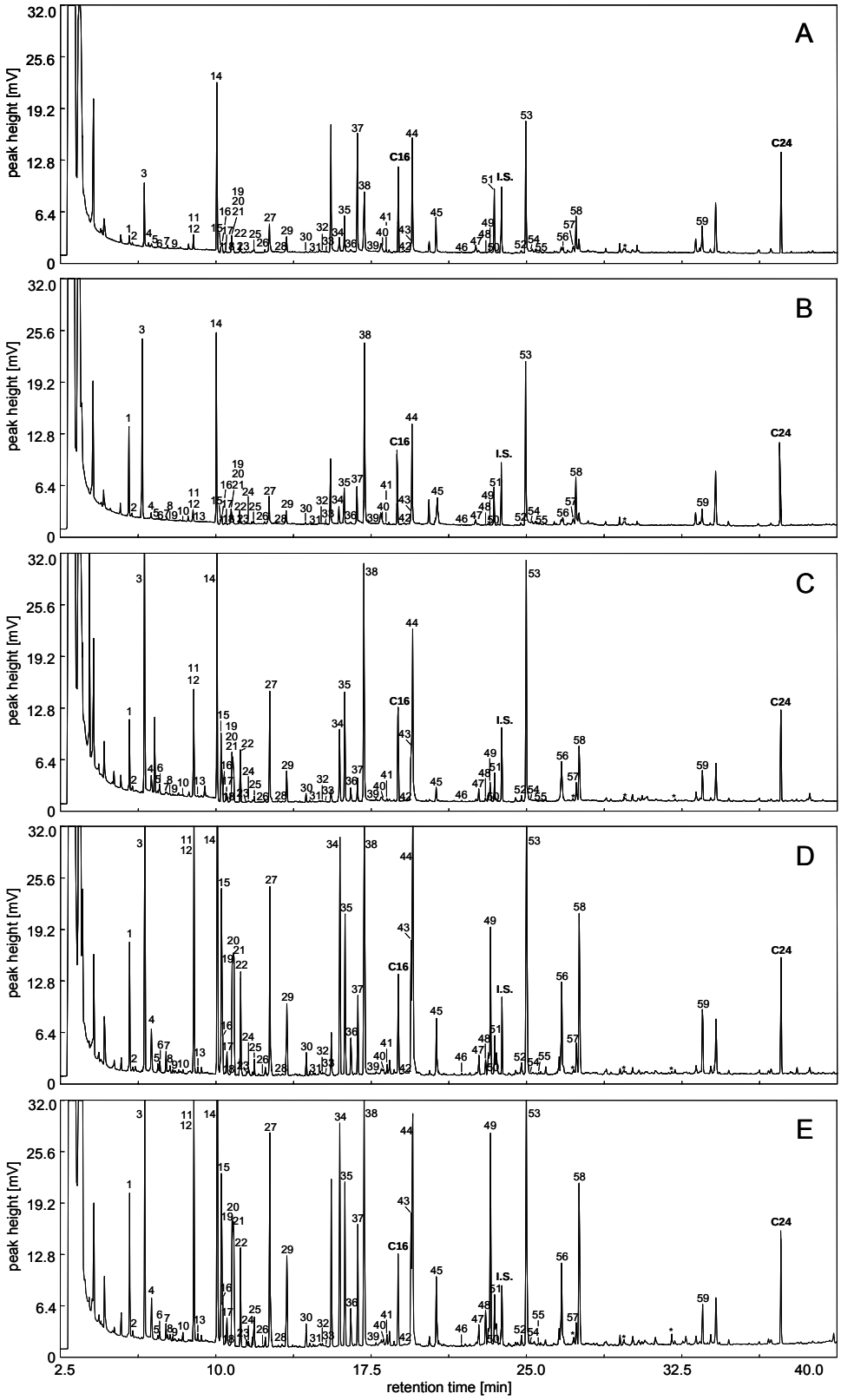

Figure 17: GC-FID chromatograms of fractions IV obtained for II32B-ge during the germination after 0h (A), 24h (B), 48h (C), 72h (D) and 96h (E). I.S.: internal standard *p*-chloro-L-phenylalanine, C16 and C18: retention time standards hexadecane and octadecane. *: residual sugars. The identification of peaks is given in Table 6.

In general, the levels of organic and inorganic acids detected in fraction IV significantly increased during the germination (Figures 15B and 16B). After an incubation time of 96h, analysis of acid changes revealed increased total levels up to +570 % in MH-ge2. Major acids in brown rice were phosphoric and citric acid representing on average 73 % of the total covered acids in fraction IV. On average, an increase of +430 % has been found for phosphoric acid in germinated rice (96 h) compared to brown rice samples. Similarly increased levels were observed for the organic acids in fraction IV.

Major free amino acids in the three ungerminated GE rice mutants were identified as aspartic acid, asparagine, phenylalanine, alanine and lysine representing on average 66 % of the total amino acid content. The levels of most amino acids increased significantly in the course of the germination. The degree of increase in the three rice samples ranged from +210 % in MH-ge3 to +570 % in MH-ge2 (Figure 15B). Representative examples of amino acids are shown in Figure 16B. For the two amino acids aspartic acid and asparagine U-shape patterns were observed. Significantly increased amino acid levels in different rice kernel fractions have also been observed after a soaking period of four hours [28]. Previous investigations of changes in the amino acid levels revealed a strong influence of the germination conditions [31, 32, 161]

Changes observed for the amines ethanolamine, γ-aminobutyric acid (GABA) and putrescine are shown in Figure 16B. A 5 to 11-fold increase in GABA content was observed for the germinated rice material. Increased contents of GABA, the biogenic amine of glutamic acid, is of particular interest because of its health-promoting impacts on blood pressure and sleeplessness and suppression of liver damage [25]. In soaked and germinated brown rice 2 to 25-fold increases in GABA contents have been reported [25, 32, 34, 35]. Accumulation of GABA during soaking and germination was shown to vary greatly depending on the rice cultivar [35] and the germination temperature [31].

The concentration of glutamic acid was shown to be significantly decreased (-76 %) in germinated brown rice [25, 32]. In contrast to these findings, the glutamic acid contents in the three analyzed brown rice kernels consistently increased during the incubation process. However, incubation conditions had been shown to have a significant impact on the changes of glutamic acid contents [32].

Compared to ungerminated rice, the levels of putrescine significantly increased in the germinated rice, particularly in the last stage of the incubation. Comparably to the findings for GABA, the levels of the precursor amino acid ornithine were found to be significantly increased rather than decreased in the germinated brown rice. Putrescine, the precursor for the polyamines spermidine and spermine, was reported to play an important role in cellular plant processes [162]. However, it is also discussed to potentiate the toxicity of other amines and may act as spoilage indicator [163]. In order to initiate the germination, the brown rice samples investigated in this study were soaked in ordinary tap water. Therefore, changes of putrescine concentration may not only be a result of endogenous but also of exogenous factors. Soaking of rice for 24 h at 35 °C was shown to increase the aerobic plate count by the factor 10^4 [32].

4.1.3 Malting of barley grains

For the investigation of germinated barley, grains obtained from the cultivar Maltasia were subjected to a micro-malting procedure as described in 3.2.4.2. Briefly, barley grains were initially soaked in tap water and subsequently incubated for a total of 144 hours. After the incubation period, the germinated barley grains were dried by means of kilning. The morphological changes of the barley kernels observed upon malting are shown in Figure 18. At the end of the incubation, shoots and roots of the grains began to form which is in agreement with the germination progress described for barley in literature [151].

Figure 18: Morphological changes of barley in the course of the malting process

4.1.3.1 Identification of barley constituents

The barley grains were subjected to the metabolite profiling procedure as described in 3.2.4 and analyzed by means of capillary gas chromatography. The metabolite profiling procedure used for the barley analysis was based on the methodology used for rice. In order to minimize the amount of sample material needed for extraction, the initial weight of barley flour was reduced compared to rice. In addition, the amount of polar extract used for fractionation was increased. GC-FID chromatograms of the non-polar and polar fractions obtained from the barley cultivar Maltasia are shown in Figure 19. GC-MS based data are given in Tables 7 and 8. 173 peaks were identified: 34 saturated / unsaturated fatty acid methyl esters and 15 hydrocarbons in fraction I, 23 free fatty acids, 9 fatty alcohols, 14 sterols / triterpenic alcohols, two phenolic compounds and two tocopherols in fraction II, 21 sugars and sugar alcohols in fraction III, 18 organic and inorganic acids, 32 amino acids / amines and three further polar compounds in fraction IV.

4.1.3.2 Principal component analysis

The applied GC metabolite profiling approach allowed the detection of a total of 587 peaks in the polar and non-polar fractions of malting barley grains which is in the same order of magnitude as those detected for germinating rice seeds.
The metabolite profiling data (peak heights and corresponding retention times) were standardized via Chrom*pare* followed by principal component analysis. As conducted for germinating rice, PCA was performed for each single fraction and additionally for the combined fractions I – IV. The scores plots obtained for the PCA are shown in Figure 20. The first two principal components PC1 and PC2 explained 56 % of the total variation in fraction I (Figure 20A); the major part of variance (42 %) was expressed by the first principal component. Regarding fraction II, 49 % of the total variance during the malting process was covered by the first two principal components (Figure 20B). Similar to the effects observed for the germination of rice, the two polar fractions exhibited a more pronounced variance in the course of malting. For fractions III and IV, 81 and 80 % of the total variance was covered by PC1 and PC2, respectively (Figures 20C and D).

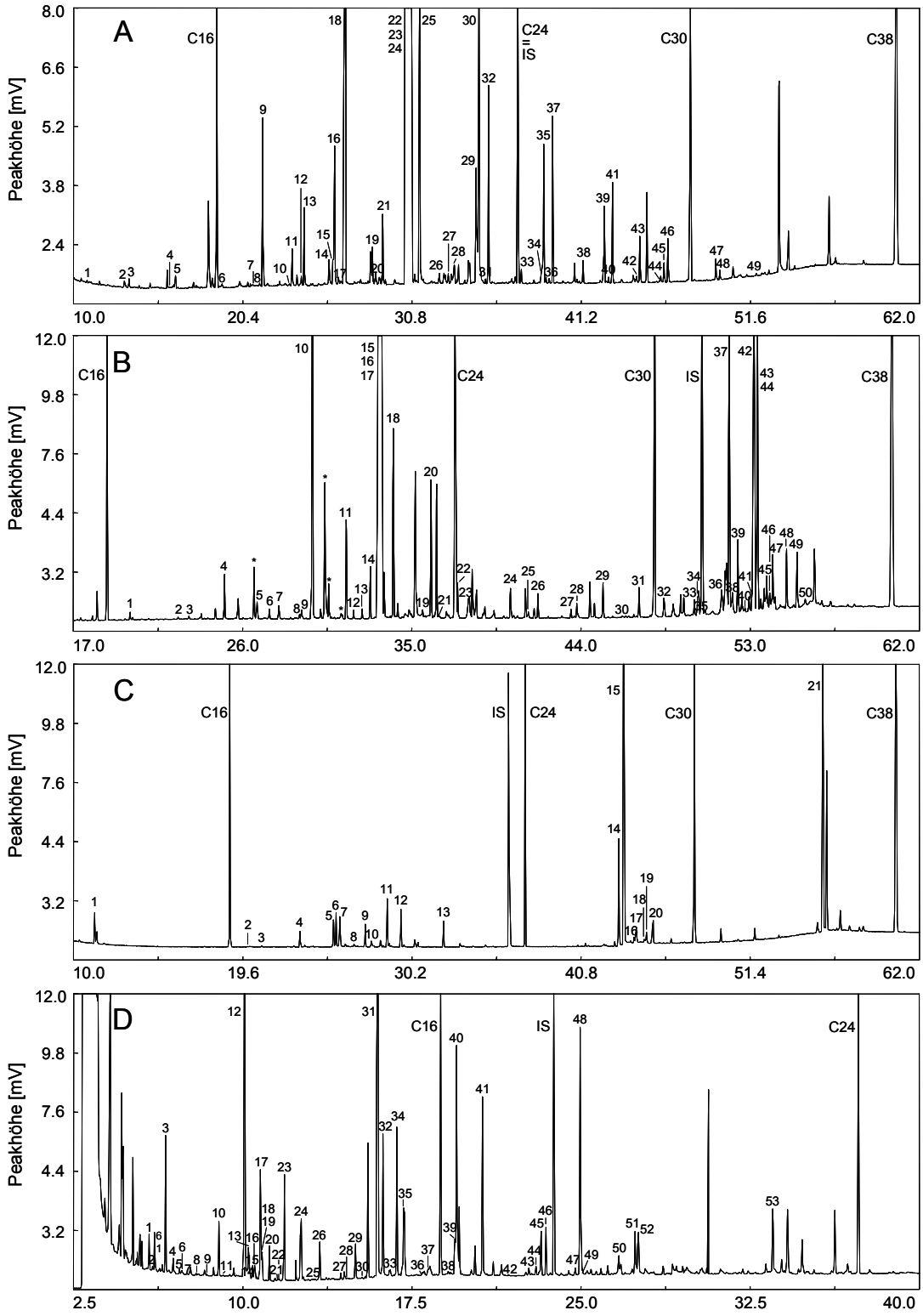

Figure 19: CG-FID chromatograms of fraction I (A), fraction II (B), fraction III (C), and fraction IV (D) obtained from barley grains. I.S.: internal standards tetradecane (A), 5α-cholestane-3ß-ol (B), phenyl-ß-D-glucopyranoside (C), *p*-chloro-L-phenylalanine (D); C16, C24, C30, C38: retention time standards; Identification of peaks is given in Tables 7 and 8. Residual FAME in fraction II and residual sugars in fraction IV are marked with asterisks.

Table 7: Compounds identified in fractions I and II of barley grains

no.	compound	ident.[a]	no.	compound	ident.[a]	no.	compound	ident.[a]
saturated FAME[b]			*unsaturated FAME*			*hydrocarbons*		
1	C10:0	A	7	C14:1	A	2	C14	A
3	C11:0	A	11	C15:1	A	4	C15	A
5	C12:0	A	15	C16:1	C	8	C17	A
6	C13:0	A	16	C16:1 cis	A	12	C18	A
9	C14:0	A	19	C17:1	A	17	C19	A
13	C15:0	A	24	C18:1	A	20	C20	A
18	C16:0	A	26	C19:1	B	27	C22	A
21	C17:0	A	30	C20:1	A	31	C23	A
25	C18:0	A	35	C22:1	A	36	C25	A
28	C19:0	A	39	C24:1	A	40	C27	A
32	C20:0	A	10	C15:2	C	42	C28	A
33	C21:0	A	14	C16:2	C	43	squalene	A
37	C22:0	A	23	C18:2	A	44	cholestane	C
38	C23:0	A	29	C20:2	A	45	C29	A
41	C24:0	A	34	C22:2	A	47	C31	A
46	C26:0	A	22	C18:3	A			
48	C28:0	A						
49	C30:0	A						
free fatty acids (1)[b]			*free fatty acids (2)*[b]			*sterols and triterpenic alcohols*[b]		
1	C12:0	A	29	C24:0	A	14	phytol	A
2	C13:0	A	32	C26:0	B	35	cholesterol	A
4	C14:0	A	36	C28:0	A	37	campesterol	A
6	C15:0	A				38	campestanol	A
8	C16:1	C	*fatty alcohols*[b]			39	stigmasterol	A
9	C16:1 (*cis* 9)	A	7	C16:0	A	40	Δ^7-campestenol	E
10	C16:0	A	13	C18:0	A	42	ß-sitosterol	A
11	C17:1	A	21	C20:0	A	43	sitostanol	A
12	C17:0	A	24	C22:0	A	44	Δ^5-avenasterol	A
15	C18:3	A	28	C24:0	C	45	gramisterol	F
16	C18:2	A	31	C26:0	A	46	cycloartenol	A
17	C18:1	A	34	C28:0	A	47	Δ^7-avenasterol	F
18	C18:0	A	41	C30:0	D	48	24-methylene-	
19	C19:1	A	50	C32:0	D		cylcoartanol	A
20	C19:0	A				49	citrostadienol	F
22	C20:1	A	*phenolic compounds*[b]					
23	C20:0	A	3	methyl *p*-OH-		*tocopherols*[b]		
25	C22:1	A		cinnamate	A	30	δ-tocopherol	A
26	C22:0	A	5	methyl ferulate	A	33	α-tocopherol	A
27	C23:0	A						

[a] Identification according to A, mass spectrometric data and retention time of reference compound; B, mass spectrometric data and retention index of the Golm metabolome database [146]; C, NIST 02 MS library; D, MS data; E, Xu and Godber 1999 [156]; F, Kamal-Eldin 1992 [157].

[b] Compounds identified as trimethylsilyl derivatives.

Table 8: TMS derivatives of compounds identified in fractions III and IV in barley grains

no.	compound	ident[a]	no.	compound	ident[a]
sugars and sugar alcohols			*amino acids and amines*		
1	glycerol	A	3	alanine	A
2,3	arabinose	A	8	2-aminobutyric acid	A
4	xylitol		9	ß-alanine	A
5,6,7	fructose	A	10	valine	A
8,10	galactose	A	13	leucine	A
9,12	glucose	A	14	ethanolamine	A
11	sorbitol		16	γ-aminobutyric acid	A
13	*myo*-inositol	A	17	isoleucine	A
14,15	sucrose	A	18	proline	A
16,17,18,19	maltose		20	glycine	A
20	trehalose	A	24	serine	A
21	raffinose	A	26	threonine	A
			27	ß-alanine	A
acids			28	homoserine	A
1	lactic acid	A	30	ß-aminoisobutyric acid	A
2	glycolic acid	A	32	pyroglutamic acid	A
5	3-hydroxypropanoic acid	C	33	methionine	A
6	pyruvic acid	A	34	aspartic acid	A
7	ß-hydroxybutyric acid	A	35	γ-aminobutyric acid	A
11	γ-hydroxybutyric acid	C	38	2-aminopimelic acid	C
12	phosphoric acid	A	39	glutamic acid	A
15	maleic acid	A	40	phenylalanine	A
19	succinic acid	A	41	asparagine	A
22	glyceric acid	A	42	α-aminoadipic acid	A
23	fumaric acid	A	44	putrescine	A
25	glutaric acid	A	45	glutamine	B
31	malic acid	A	47	citrulline	A
36	threonic acid	A	49	ornithine	A
37	3-phenyl lactic acid	A	50	histidine	A
43	*cis*-aconitic acid	C	51	lysine	A
46	2-aminoethylphosphoric acid	C	52	tyrosine	A
48	citric acid	A	53	tryptophan	A
others					
4	2-pyrrolidinone	A			
21	2,4-hydroxypyrimidine	C			
29	parabanic acid	C			

[a] Identification according to A, mass spectrometric data and retention time of reference compound; B, mass spectrometric data and retention index of the Golm metabolome database [146]; C, NIST 02 MS library.

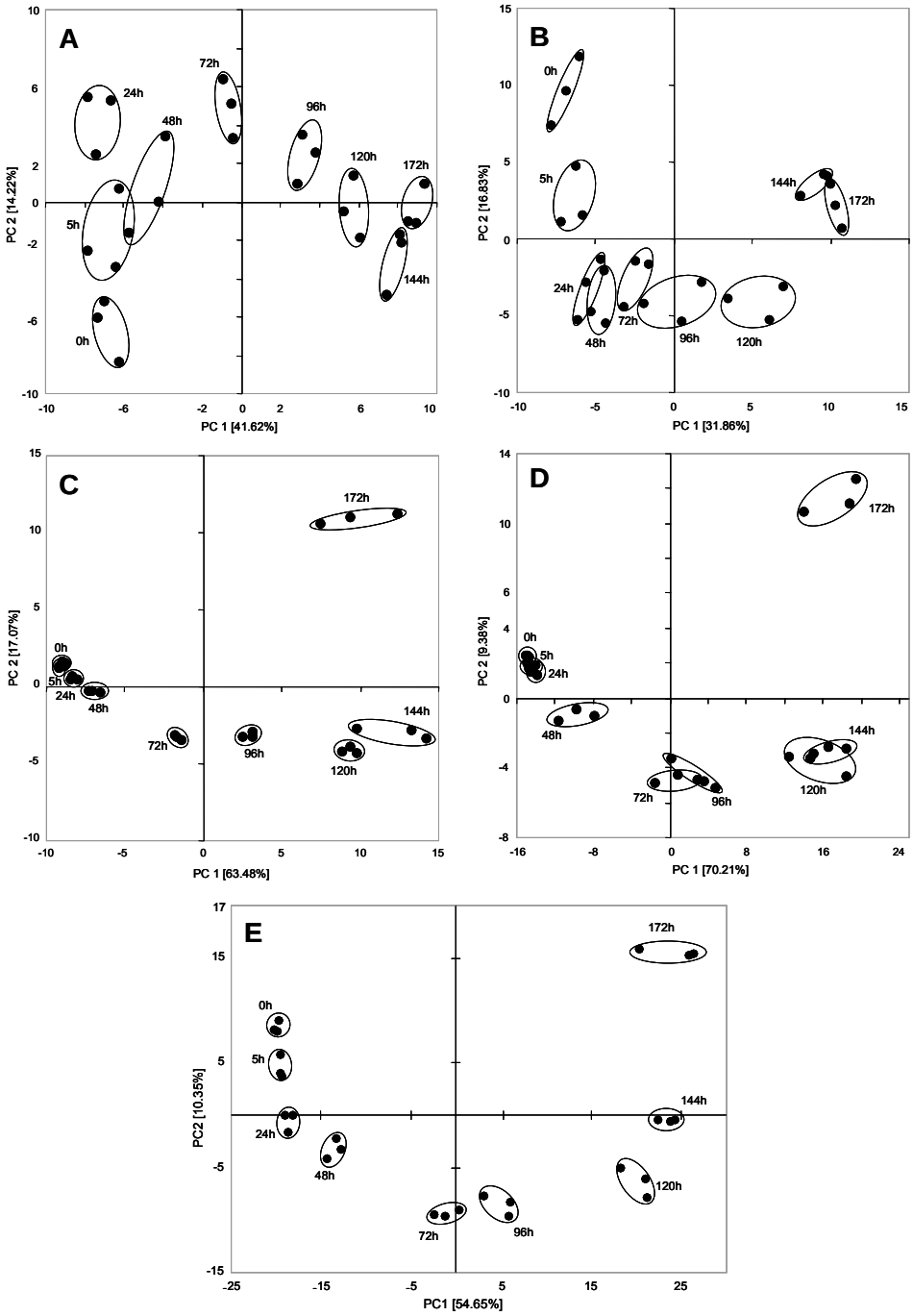

Figure 20: Principal component analysis of standardized GC-FID metabolite profiling data of fractions I (A), II (B), III (C) and IV (D), and of combined fractions I - IV (E) in the course of malting (0, 5, 24, 48, 72, 96, 120, 144, 172 h) of the barley cultivar Maltasia

The germinated and kilned barley samples (172 h) in fractions III and IV were clearly separated from the rest of the samples. As shown in Figure 20E, the PCA plot considering the combined polar and non-polar fractions reflects the more pronounced influence of the polar constituents on the separation of germinating barley during the

malting procedure. The metabolic-related variance of malting barley covered by principal component analyses (PC1 and PC2) was shown to be similar to those found for germinating rice (4.2.2) and tomato seeds and fruits during different developmental stages [17, 116].

4.1.3.3 Relative quantification of compounds

The quantification of compounds in the course of malting was performed on the basis of standardized GC peak heights. The dynamic changes of selected major fatty acid methyl esters (FAME) and hydrocarbons obtained from fraction I (major lipids) are shown in Figure 21. The germinated barley grains exhibited a slight decrease of total FAME by -5 %, whereas the hydrocarbons were shown to be significantly increased during the malting.

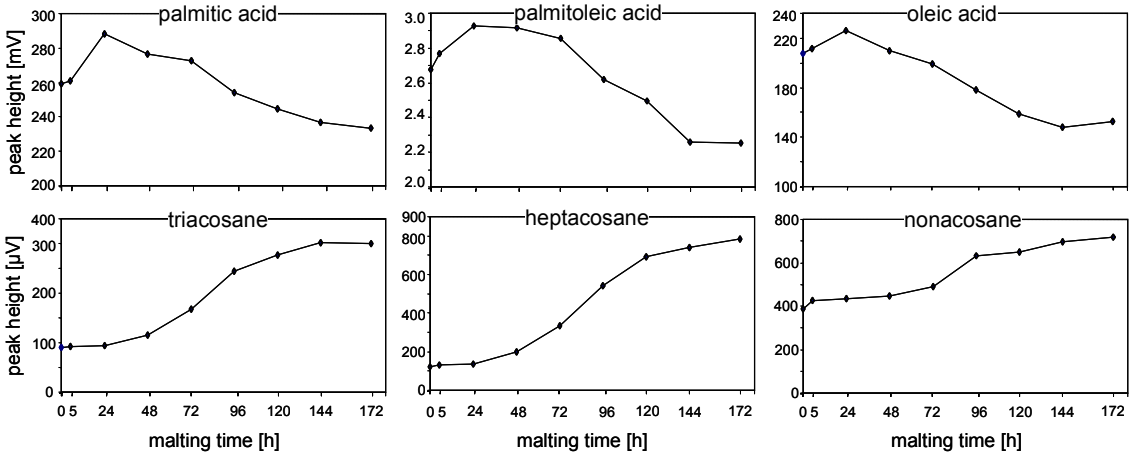

Figure 21: Standardized peak heights of selected fatty acid methyl esters and hydrocarbons obtained from fraction I during the malting process.

A decrease of the crude lipid content during germination by -25% has been reported for barley [151]. However, it was also noted, that the degree of lipid degradation is greatly influenced by the germination conditions, e.g. temperature, moisture and germination time. Similar to germinated rice, barley revealed significantly increased hydrocarbon contents which might result from an improved extractability of this compound class due to an enzymatic degradation of lipid-containing starch.

The overall changes of the metabolites present in fraction II (minor lipids) are relatively small. At the initial phase of germination (until 48h), the concentrations of

free fatty acids decreased rapidly. After an incubation time of 48h, levels of free fatty acids were shown to be more gently decreased. Considering the fatty alcohols, no consistent trend has been observed in the course of germination which is in agreement with observations made for germinating rice. Comparable to rice, the contents of the major sterols β-sitosterol, campesterol and stigmasterol increased approximately +25 % in the malt compared to the ungerminated barley grains. In soybeans these sterols exhibited an average increase of +30 % after 120 hours of germination which is very similar to those observed in the present study [164]. The changes of the sterols stigmasterol and β-sitosterol and additionally of stearic acid are shown in Figure 22.

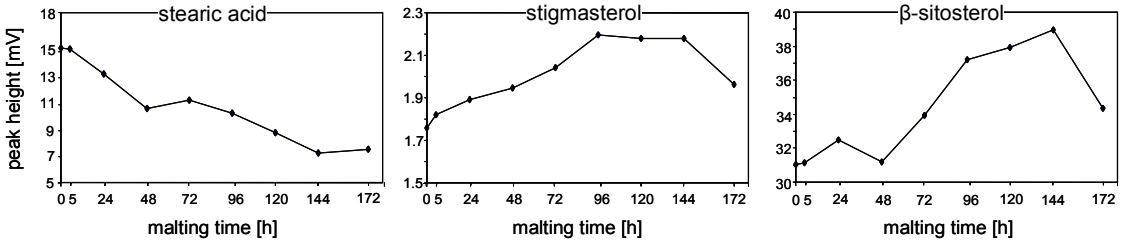

Figure 22: Standardized peak heights of free stearic acid, stigmasterol and β-sitosterol obtained from fraction II during the malting process.

Compared to the dynamic changes of lipids, polar compounds exhibited much more pronounced alternations during germination. The dynamic changes of standardized peak heights determined in the polar fractions III and IV are shown in Figure 23.

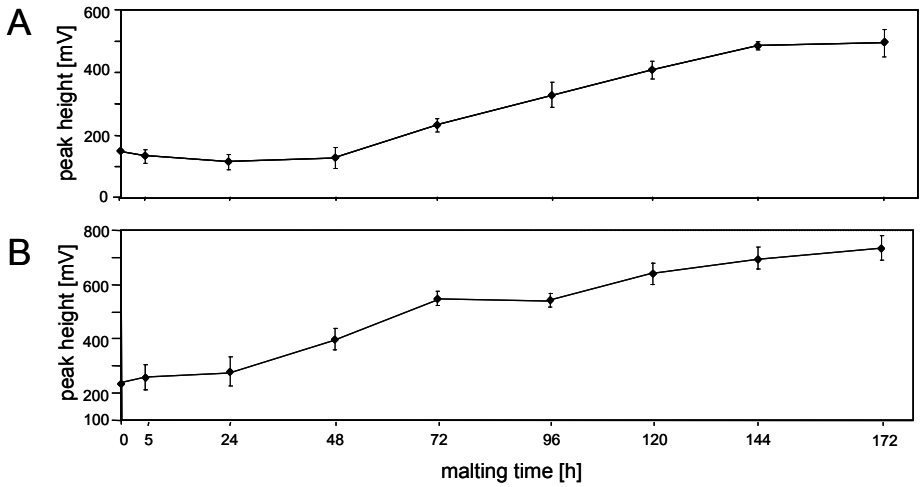

Figure 23: Total standardized peak heights of metabolites in the polar fractions III (A) and IV (B) obtained from malted barley grains

Levels of total peak heights from both fractions increased significantly during the malting process by +230 and +200%, respectively. For fraction III (sugars), significantly increased levels of mono- and disaccharides, e.g. glucose and maltose were observed (Figure 24). After kilning (172h), the concentrations of fructose and glucose decreased as a result of the formation of maillard products at higher temperatures. In contrast to the mono- and disaccharides, the level of the trisaccharide raffinose was shown to be significantly reduced in the course of barley malting (Figure 24). Raffinose acts as donor for the provision of energy supply at the start of germination. A similar effect has been observed in germinating rice and has been described in literature for germinating barley [151].

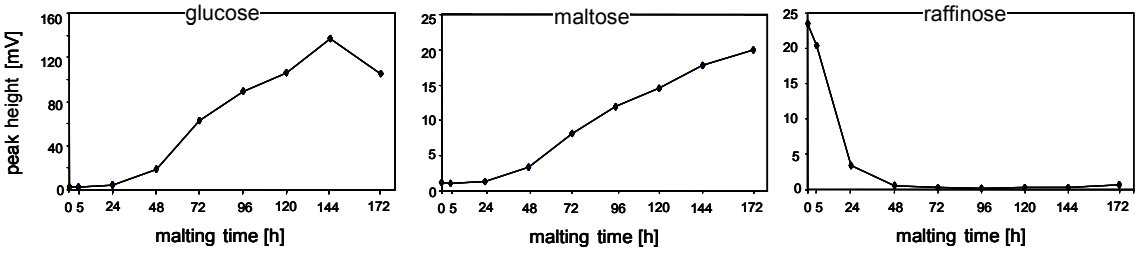

Figure 24: Standardized peak heights of glucose, maltose and raffinose obtained from fraction III during the malting process.

Representative changes for selected compounds in fraction IV (acids, amino acids) are shown in Figure 25. The levels of the amino acids serine, asparagine and glutamine increased by up to +2000% during germination. Higher levels of amino acids (up to +800%) have also been reported for germinated wheat [161]. However, after kilning of the germinated barley grains, contents of amino acids in malt (172h) were lower than in the germinated grains (144h). Similar to the expected changes observed for reducing sugars during the kilning process, a formation of maillard products led to the decrease of amino acids in fraction IV. In contrast to the observations made for asparagine and aspartic acid in germinating rice, the U-shape patterns of these two compounds in rice could not be confirmed in germinating barley.

Malic, phosphoric and citric acid are the major acids in fraction IV. The levels of fumaric, phosphoric and citric acid were strongly increased in germinated barley (Figure 25).

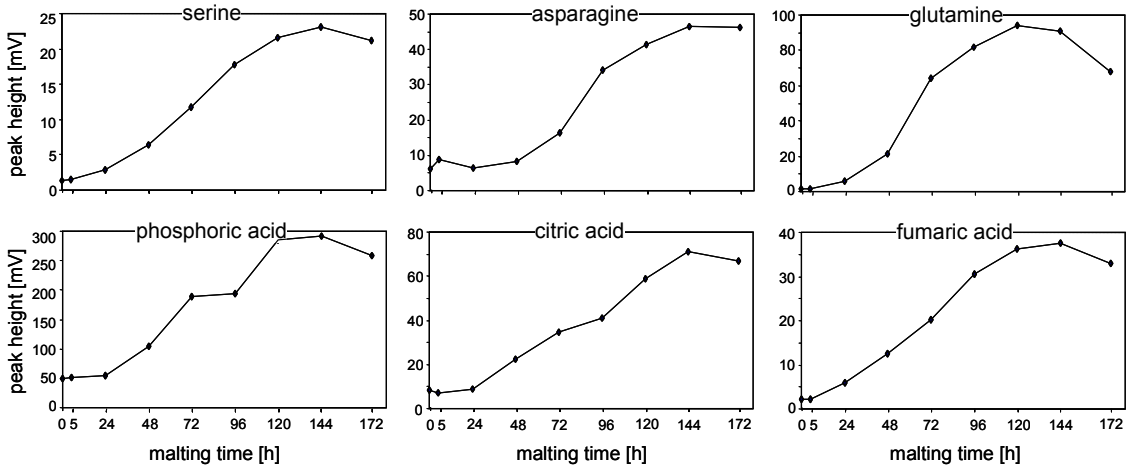

Figure 25: Standardized peak heights of selected amino acids and organic / inorganic acids obtained from fraction I during the malting process.

Investigation of organic acids in barley during malting also revealed increased acid levels after six days of germination [165]. The overall changes were much less pronounced than those observed in the present study. However, it has to be considered, that the germination parameters, e.g. temperature and incubation moisture may have a great influence on the crop metabolites.

4.1.4 Conclusions

The data obtained demonstrate the suitability of the described metabolite profiling technique to follow metabolic changes in a complex plant matrix. Owing to inherent features of the approach, e.g. choice of extraction solvents, derivatization steps or volatilities of derivatives, the type of metabolites covered is to some extent pre-determined and not fully “unbiased”. However, the metabolites stem from a wide range of chemical classes and the number of compounds detected and identified is in an order of magnitude comparable to those reported for other GC-MS based metabolite profiling studies [8, 14]. In addition to representatives of primary plant metabolism, nutritionally relevant metabolites are covered. They range from the lipophilic sterols to the polar amino compounds γ-aminobutyric acid and putrescine. The applied methodology is suitable to cover those metabolites shown to form a basis for metabolic phenotyping of rice variants in a GC-MS approach [10]. The

metabolic changes observed during the germination and malting of rice and barley grains also confirm the potential of metabolite profiling techniques to reveal distinct metabolic switches as indicated for the development and germination of Arabidopsis seeds [15].

Further studies are required to determine whether the time-dependent score patterns observed for the rice and the barley materials investigated in this study are also valid for other species and incubation conditions. Ideally, a set of biomarker metabolites could be developed representing the germination period, as shown for the initiation and early growth of rice tillering [14]. Such marker metabolites might also be correlated to the contents of nutritionally important metabolites claimed to be responsible for the advantageous properties of germinated crops [25].

4.2 INVESTIGATION OF LOW PHYTIC ACID RICE MUTANTS

4.2.1 Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate, $InsP_6$) represents a major anti-nutrient in food and feed. In seeds, it is stored as mixed salts of cations, mainly K and Mg, together with minor amounts of Ca, Fe, Zn and Mn [47]. Owing to the formation of indigestible chelates, the bioavailability of nutritionally relevant minerals may be limited [70]. For example, lowered absorption of iron and zinc from diets rich in phytic acid is discussed as a major factor for iron and zinc deficiencies in developing countries where well-balanced diets are not available and people rely on staple foods such as rice or legumes [71].

Owing to these disadvantageous effects, various efforts have been made to breed crop varieties low in phytic acid content. Mutation breeding has been successfully applied to generate low phytic acid (*lpa*) mutants of maize [94-96], barley [97, 98], rice [92, 99], soybean [93, 102, 103] and wheat [101]. Three different types of *lpa*-mutations have been described in maize: The first reported *lpa*-1 mutants are characterized by a decrease in phytic acid content and a corresponding increase in inorganic phosphorus [51, 92, 94]. In *lpa*-2 mutants the decrease in phytic acid content is accompanied by an accumulation of lower inositol phosphates [94, 98]. In *lpa*-3 mutants no lower inositol phosphates were detected but an increase in *myo*-inositol content was described [59, 100]. Downregulation of genes encoding enzymes considered as essential for the biosynthesis of phytic acid, i.e. *myo*-inositol phosphate synthase or *myo*-inositol kinase, resulted in characteristic changes in the levels of certain crop metabolites [59, 103, 166].

The study objects of this investigation were three *lpa* rice mutants (*Os*-*lpa*-XQZ-1, *Os*-*lpa*-XS110-1 and *Os*-*lpa*-XS110-2) generated by γ-irradiation [99]; the nature of the mutations underlying the decrease in phytic acid was not known. The aim was to compare these *lpa* rice mutants to the corresponding wild-type by means of metabolite profiling and to explore the usefulness of this approach to assist in the elucidation of the types of mutation resulting in the reduced content of phytic acid.

4.2.2 Analysis of inositol phosphates

Inositol phosphates isolated from the rice materials were analyzed by ion-pair HPLC. Table 9 shows the data obtained for the wild-types Xieqingzao B and Xiushui 110 and the *lpa* mutants *Os-lpa*-XQZ-1, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 grown at six field trials over two growing seasons in China. The phytic acid contents in the wild-type rice ranged from 0.71 to 1.04 % in Xieqingzao B and from 0.73 to 1.12 % in Xiushui 110 and differed depending on the growing location. Lower inositol phosphates ($InsP_3$, $InsP_4$) were not detected, except for marginal amounts of $InsP_5$ (max. 0.08 %). The different phytic acid contents determined in the wild-types depending on the field trial confirm the environmental impact. This effect is in agreement with data reported for other conventional rice cultivars [49, 167].

Table 9: Contents of phytic acid in the rice wild-types Xieqingzao B and Xiushui 110 and the corresponding mutant lines *Os-lpa*-XQZ-1, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2.

Field trial	Phytic acid [% dry matter]				
	Xieqingzao B	*Os-lpa*-XQZ-1	Xiushui 110	*Os-lpa*-XS110-1	*Os-lpa*-XS110-2
2003/2004					
Hainan	1.04 ± 0.04	0.68 ± 0.02	1.08 ± 0.06	0.49 ± 0.00	0.84 ± 0.03
Jiaxing	0.80 ± 0.01	0.56 ± 0.02	0.73 ± 0.00	0.45 ± 0.00	0.51 ± 0.01
Hangzhou ZAAS[1]			0.77 ± 0.05	0.51 ± 0.02	0.68 ± 0.01
Hangzhou ZJU[2]			1.06 ± 0.01	0.53 ± 0.03	0.78 ± 0.03
2005/2006					
Hainan	0.85 ± 0.01	0.75 ± 0.02	0.87 ± 0.01	0.42 ± 0.02	0.68 ± 0.03
Jiaxing	0.71 ± 0.02	0.50 ± 0.01	0.73 ± 0.03	0.45 ± 0.01	0.55 ± 0.02
Hangzhou ZAAS	0.97 ± 0.01	0.65 ± 0.01	0.90 ± 0.01	0.44 ± 0.03	0.79 ± 0.01
Guangzhou	0.84 ± 0.01	0.65 ± 0.02	1.04 ± 0.00	0.54 ± 0.01	0.93 ± 0.03
Fuzhou	0.96 ± 0.00	0.73 ± 0.00	1.12 ± 0.01	0.66 ± 0.00	0.78 ± 0.03

1 ZAAS Zhejiang Academy of Agricultural Sciences

2 ZJU Zhejiang University

Compared to the wild-types, the *lpa* mutants exhibited significantly lower contents in phytic acid. The extents of phytic acid reduction were different for the mutant lines ranging from -12 to -35 % for *Os-lpa*-XQZ-1, from -34 to -58 % for *Os-lpa*-XS110-1 and from -11 to -30 % for *Os-lpa*-XS110-2. Overall, the reduction in phytic acid

content was consistently more pronounced in *Os-lpa*-XS110-1 (on average -46 %) than in *Os-lpa*-XQZ-1 (-27 %) and *Os-lpa*-XS110-2 (-22 %) which is in agreement with data reported for other locations [99]. In addition, it has been shown for these locations, that the levels of total phosphorus in the rice wild-types and the corresponding *lpa* mutants are similar. Accumulations of lower inositol phosphates were not detected for the *lpa* rice mutants.

In order to evaluate the natural variability of phytic acid contents in the rice wild-types and low phytic acid mutants, a set of conventional rice samples comprising the two varieties *indica* and *japonica* (*n*=48), red and black rice (*n*=15) and various commercial samples from Italy, Spain, India and the USA (*n*=8) was investigated by ion-pair HPLC (Figure 26). The phytic acid contents in this set of brown rice samples ranged from 0.64 to 1.73 %. As shown for the two wild-types Xieqingzao B and Xiushui 110, the broad ranges of phytic acid contents in the conventional samples confirm the impact of grain characteristics and environmental influence.

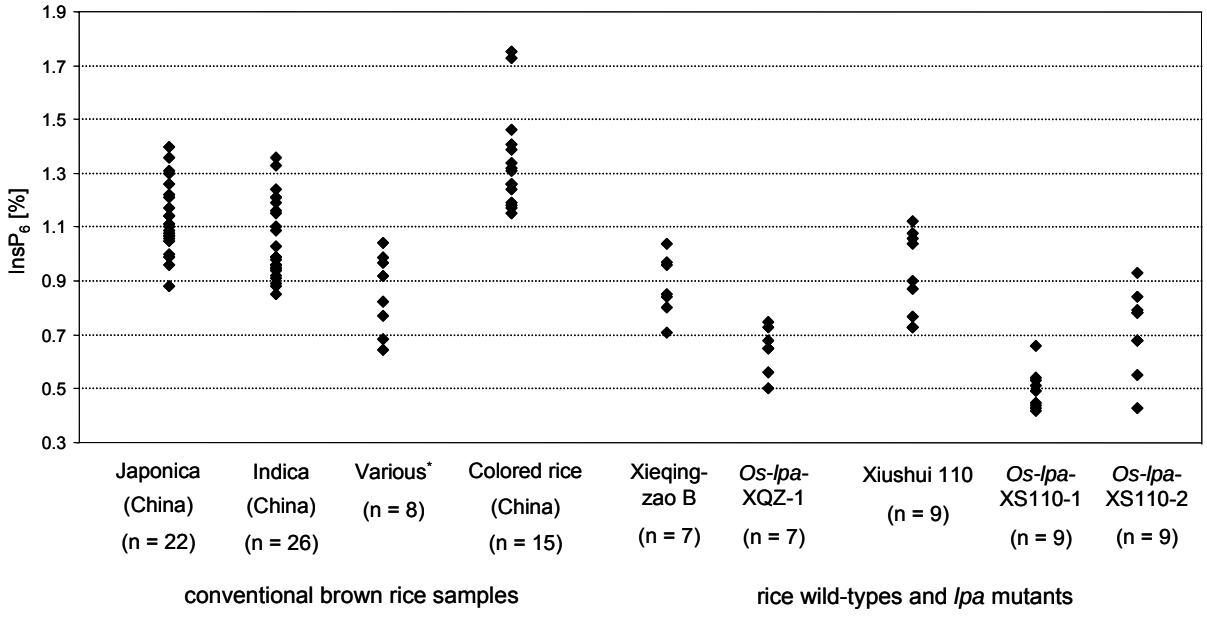

Figure 26: Contents of phytic acid in conventional brown rice, low phytic acid mutants and corresponding wild-types. * Brown rice samples from Italy (*n*=4), Spain (*n*=2), India (*n*=1) and USA (*n*=1).

4.2.3 Contents of divalent cations

4.2.3.1 Contents of calcium, iron and zinc

The *indica lpa* rice mutant *Os-lpa*-XQZ-1 exhibited statistically significantly higher levels of calcium (average: +20 %), iron (+16 %) and zinc (+19 %) compared to the corresponding wild-type at all growing locations and during all seasons investigated (Table 10). The results are in agreement with the observations made for this mutant in a recent study with material from three other field experiments [167]. They provide further experimental support that this specific rice mutant has the general potential to accumulate mineral elements. However, it cannot be finally concluded whether these observations result from the intended mutation, from pleiotropic effects of the target gene or further mutations independent from the primary mutation event. For example, microsatellite analysis of γ-irradiated rice has revealed that out-crosses are responsible for different plant phenotypes [168, 169]. From a nutritional point of view it is noteworthy that both the mean contents of calcium, iron and zinc in the wild-type rice as well as the increased contents in the mutant were within the reported natural range [170, 171].

The data obtained confirm the pronounced environmental impact on the cation levels. Although the contents of calcium, iron and zinc are statistically significantly higher compared to the wild-type at each individual field trial (except for calcium at location Fuzhou, 2005), the lowest content of each element in the mutant *Os-lpa*-XQZ-1 was still lower than the highest content in the wild-type across the seven field trials. For calcium and zinc these results are in agreement with the recently conducted study on *Os-lpa*-XQZ-1 at three other field trials [167]. For iron, it had been found in the former study that even the lowest content in the *lpa* mutant was still higher than the highest content in the wild-type grown at the three field trials. However, this observation could not be confirmed under the conditions of the present field trials. Compared to the calcium concentration in the *japonica* wild-type XS110 (average: 140 mg/kg), no consistent changes were observed for *Os-lpa*-XS110-1 (average: 142 mg/kg), whereas *Os-lpa*-XS110-2 (average: 153 mg/kg) exhibited consistently elevated calcium levels (Table 10).

Table 10: Contents of calcium, iron and zinc in the lpa mutants *Os-lpa*-XQZ-1, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 and corresponding wild-types Xieqingzao B and Xiushui 110.

growing location	Xieqing-zao B	*Os-lpa*-XQZ-1	Xiushui 110	*Os-lpa*-XS110-1	*Os-lpa*-XS110-2
	calcium [mg/kg]				
2003/2004					
Hangzhou ZAAS[1]	-	-	125 ± 1	129 ± 1	139 ± 4
Hangzhou ZJU[2]	-	-	161 ± 3	151 ± 2	172 ± 2
Hainan	105 ± 3	125 ± 0	-	-	-
Jiaxing	120 ± 1	137 ± 2	-	-	-
2005/2006					
Hangzhou ZAAS	130 ± 1	170 ± 3	159 ± 2	154 ± 3	164 ± 2
Hainan	130 ± 1	180 ± 2	153 ± 1	148 ± 1	159 ± 1
Jiaxing	106 ± 4	130 ± 2	123 ± 2	114 ± 1	133 ± 2
Guangzhou	126 ± 2	180 ± 1	125 ± 3	147 ± 2	142 ± 1
Fuzhou	125 ± 3	126 ± 3	135 ± 2	149 ± 2	161 ± 1
	iron [mg/kg]				
2003/2004					
Hangzhou ZAAS[1]	-	-	14.9 ± 0.2	17.8 ± 0.4	14.0 ± 0.6
Hangzhou ZJU[2]	-	-	17.8 ± 0.6	18.3 ± 0.6	22.7 ± 0.5
Hainan	13.3 ± 0.7	15.3 ± 0.6	-	-	-
Jiaxing	10.5 ± 0.2	12.4 ± 0.1	-	-	-
2005/2006					
Hangzhou ZAAS	14.0 ± 0.2	17.6 ± 0.4	13.2 ± 0.1	13.8 ± 0.3	13.1 ± 0.5
Hainan	16.9 ± 0.7	19.3 ± 0.6	15.4 ± 0.4	16.1 ± 0.2	14.4 ± 0.5
Jiaxing	12.5 ± 0.7	14.7 ± 0.4	13.3 ± 0.5	13.0 ± 0.3	11.4 ± 0.2
Guangzhou	12.1 ± 0.2	15.5 ± 0.7	14.7 ± 0.4	13.5 ± 0.2	13.5 ± 0.1
Fuzhou	14.5 ± 0.2	17.5 ± 0.4	13.7 ± 0.3	13.8 ± 0.4	12.9 ± 0.1
	zinc [mg/kg]				
2003/2004					
Hangzhou ZAAS[1]	-	-	19.3 ± 0.3	17.3 ± 0.2	23.3 ± 1.0
Hangzhou ZJU[2]	-	-	29.3 ± 0.5	28.1 ± 0.1	35.8 ± 0.5
Hainan	29.8 ± 0.9	32.3 ± 0.1	-	-	-
Jiaxing	17.1 ± 0.3	20.2 ± 0.4	-	-	-
2005/2006					
Hangzhou ZAAS	22.7 ± 0.3	29.9 ± 0.5	23.6 ± 0.3	19.4 ± 0.2	27.8 ± 0.3
Hainan	23.7 ± 0.1	31.8 ± 0.6	24.4 ± 0.1	20.1 ± 0.1	27.4 ± 0.4
Jiaxing	22.0 ± 0.7	28.6 ± 0.4	26.2 ± 0.4	22.2 ± 0.2	26.1 ± 0.2
Guangzhou	25.2 ± 0.5	29.5 ± 1.5	31.9 ± 0.4	26.3 ± 0.2	28.0 ± 0.2
Fuzhou	24.8 ± 0.6	31.6 ± 0.5	22.1 ± 0.2	22.8 ± 0.1	27.9 ± 1.0

Determination of iron in *Os-lpa*-XS110-1 (average: 15.2 mg/kg) and *Os-lpa*-XS110-2 (average: 14.6 mg/kg) revealed no consistent changes in the mutants compared to the wild-type (average: 14.7 mg/kg). Zinc concentrations in XS110 ranged from 19.3 to 31.9 mg/kg. *Os-lpa*-XS110-1 exhibited predominantly decreased zinc concentrations (average: -14 %) compared to the wild-type. The zinc contents in *Os-lpa*-XS110-2 were mostly increased, but again the changes were not consistent over all field trials. The inconsistent patterns observed for *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 are in agreement with previous observations [167]. The data obtained for the wild-type and the two *japonica lpa* rice mutants reflect a pronounced environmental impact on the cation levels. Based on the observations made in this study, there is no obvious correlation between the decreased contents of phytic acid in *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 and the inconsistently altered levels of divalent cations.

4.2.3.2 Contents of cadmium

The analysis of cadmium in the rice wild-types revealed considerable variations in concentrations depending on the growing location and the season indicating a large impact of the environmental conditions (Table 11). This effect is in agreement with data reported for cadmium in conventional rice [172-174]. Cadmium contents in the *indica* wild-type XQZ ranged from 16 to 104 µg/kg. On average, the cadmium concentration was decreased in *Os-lpa*-XQZ-1 (48 µg/kg) compared to the wild-type (60 µg/kg). However, at three of seven field trials the cadmium concentrations in wild-type and *lpa* mutant were within the same order of magnitude. Decreased cadmium levels in *Os-lpa*-XQZ-1 were only observed when the cadmium concentrations in the wild-type were high. The results indicate that *Os-lpa*-XQZ-1 might have the potential to decrease the cadmium concentration in the plant when the cadmium load in the soil is high. Information on the cadmium contaminations in the growing areas was not available. Therefore, these findings need to be confirmed in further studies that should include analyses of the corresponding soil samples. The set of analytes might be extended by lead, another contaminant often associated with cadmium.

Table 11: Cadmium contents in the *lpa* mutants *Os-lpa*-XQZ-1, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 and corresponding wild-types Xieqingzao B and Xiushui 110.

growing location	cadmium [µg/kg]				
	Xieqing-zao B	*Os-lpa*-XQZ-1	Xiushui 110	*Os-lpa*-XS110-1	*Os-lpa*-XS110-2
2003/2004					
Hangzhou ZAAS[1]	-	-	127 ± 5	24 ± 0	36 ± 1
Hangzhou ZJU[2]	-	-	150 ± 6	154 ± 8	158 ± 4
Hainan	104 ± 1	85 ± 2	-	-	-
Jiaxing	16 ± 0	16 ± 1	-	-	-
2005/2006					
Hangzhou ZAAS	40 ± 2	44 ± 1	63 ± 1	53 ± 1	130 ± 1
Hainan	42 ± 1	45 ± 1	59 ± 0	53 ± 1	118 ± 1
Jiaxing	79 ± 5	60 ± 2	50 ± 1	32 ± 1	53 ± 0
Guangzhou	60 ± 2	19 ± 0	28 ± 1	14 ± 1	19 ± 1
Fuzhou	80 ± 2	64 ± 3	152 ± 3	93 ± 1	130 ± 3

Cadmium contents in the *japonica* wild-type XS110 ranged from 28 to 152 µg/kg. Compared to the wild-type (average: 90 µg/kg), the *lpa* mutant *Os-lpa*-XS110-1 (60 µg/kg) exhibited predominantly lowered cadmium concentrations (Table 11). A consistent trend was not observed for *Os-lpa*-XS110-2 (19 to 158 µg/kg). Considering the inconsistent results obtained for cadmium contents in the *japonica* rice mutant, there is no evidence for a potential correlation between the extent of phytic acid reduction and the altered cadmium concentrations in the *lpa* mutants.

4.2.3.3 Molar ratios of phytic acid and minerals

Phytate is a known inhibitory factor for mineral absorption in animals and humans [70]. The degree of inhibition depends on the phytate and on the element concentrations in food and feed. The relative bioavailability of minerals in humans was suggested to be expressed as molar ratios of phytate / minerals [175-177]. Lower phytate / mineral molar ratios implied improved bioavailability of nutritionally important minerals. The predicted limit values for adequate mineral absorption in humans were < 0.24 for phytate / calcium [175], < 1 for phytate / iron [176] and < 15 for phytate / zinc [177].

The phytate / mineral molar ratios for the *indica* and *japonica lpa* rice and the corresponding wild-types grown in the season 2005/06 are shown in Figure 27. Compared to wild-types, the *indica lpa* mutant *Os-lpa*-XQZ-1 and the *japonica lpa* mutant *Os-lpa*-XS110-1 exhibited statistically significantly lowered phytate / mineral molar ratios for all analyzed minerals. Despite improved phytate / mineral molar ratios determined for *Os-lpa*-XS110-2, none of them was statistically significantly different ($p < 0.05$) compared to the wild-type XS110. The decreased phytate / mineral molar ratios for the *lpa* rice mutants were mainly based on the decreased phytic acid contents. The degree of phytic acid reduction was consistently more pronounced in *Os-lpa*-XS110-1 than in *Os-lpa*-XQZ-1 and *Os-lpa*-XS110-2, resulting in a decrease of phytate / mineral molar ratios in *Os-lpa*-XS110-1. Reductions in phytic acid in *Os-lpa*-XQZ-1 and *Os-lpa*-XS110-2 were within the same order of magnitude. However, owing to the consistently increased seed mineral contents, only *Os-lpa*-XQZ-1 exhibited statistically significantly improved molar ratios for all analyzed minerals compared to the wild-type.

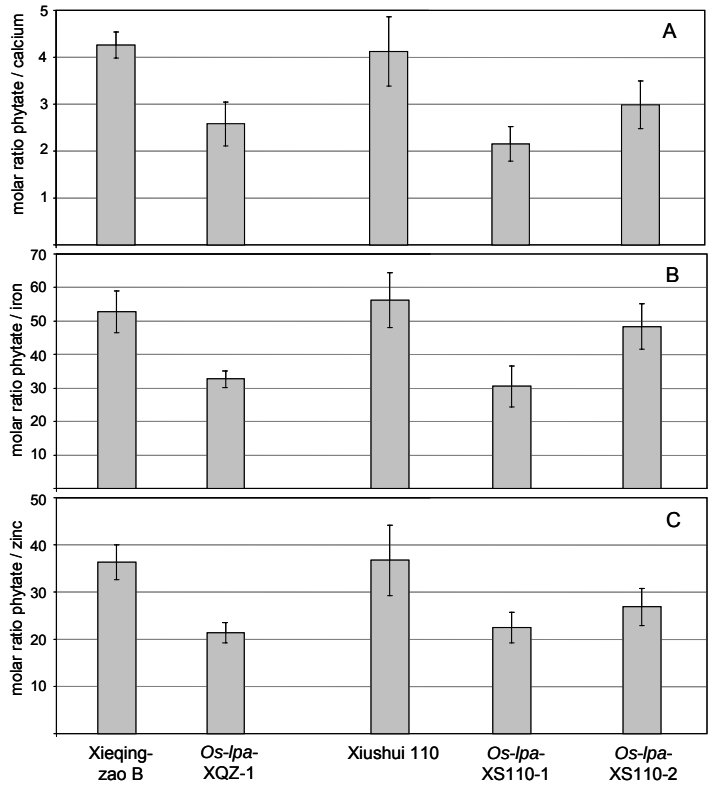

Figure 27: Molar ratios of phytate / calcium (**A**), phytate / iron (**B**) and phytate / zinc (**C**) in *indica* and *japonica lpa* rice mutants and wild-types grown at five field trials in 2005/06 (Hainan, Jiaxing, Hangzhou ZAAS, Guangzhou, Fuzhou) (mean ± confidence interval, $n = 5$, $p < 0.05$)

The molar ratios of phytate / calcium for all *lpa* mutants still indicate limitations of the bioavailability of calcium considering the predicted molar ratio of 0.24 and above for impaired calcium absorption in humans [175]. Despite reduced phytate / iron molar ratios in all *lpa* rice mutant lines, iron bioavailability may still be limited. However, the *lpa* mutants *Os-lpa*-XQZ-1 and *Os-lpa*-XS110-1 exhibited improved phytate / iron molar ratios on brown rice basis compared to literature data for milled rice [178, 179]. As regards the phytate / zinc molar ratios, both, wild-types and *lpa* mutants exceed the range of sufficient zinc provision [177].

4.2.4 Metabolite profiling of low phytic acid rice mutants

4.2.4.1 Comparative analysis of wild-types and mutants

Metabolite profiling was performed according to the extraction and fractionation procedure described in 3.2.3. Briefly, lipids and polar compounds were consecutively extracted from the freeze-dried rice flour. Lipids were transesterified in methanol and subsequently separated by solid phase extraction into a fraction containing fatty acid methyl esters (fraction I) and a fraction containing the minor lipids (fraction II). Selective hydrolysis of silylated derivatives was applied to separate the polar extract into a fraction containing silylated sugars and sugar alcohols (fraction III) and a fraction containing organic acids and amino acids (fraction IV). The four fractions obtained were analyzed by gas chromatography (GC-FID). Peak heights and corresponding retention times were exported to Chrom*pare*, a software tool developed for comparative analysis of metabolite profiling data [147] (www.chrompare.com). Chrom*pare* automatically corrects retention time shifts on the basis of retention time standards and standardizes peak heights on the basis of internal standards added before the fractionation process. Comparison of metabolite profiles is performed by comparison of mean standardized peak heights based on triplicate analysis. Means are considered as statistically significant different if their confidence intervals ($p < 0.05$) are not overlapping. Results obtained by comparative metabolite profiling of the wild-types Xieqingzao B and Xiushui 110 and mutant lines *Os-lpa*-XQZ-1, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 grown in 2003/2004 and 2005/2006 are shown in Tables 12 and 13.

Table 12: Peak-based comparison of chromatograms obtained by metabolite profiling of wild-types Xieqingzao B and Xiushui 110 and mutant lines *Os-lpa*-XQZ-1, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 grown in 2003/2004.

		field trial								
		Hainan		Jiaxing		Hangzhou 1		Hangzhou 2		
fraction	compound class	total[a]	diff.[b]	total	diff.	total	diff.	total	diff.	consistent differences[c]
Xieqingzao B vs. Os-lpa-XQZ-1										
I	FAME	19	0	26	3					-
II	minor lipids	43	6	39	0					-
III	sugars	9	0	9	0					-
IV	acids, AS	18	4	23	8					1
Σ I - IV		89	10	97	11					1
Xiushui 110 vs. Os-lpa-XS110-1										
I	FAME	30	10	27	1	26	8	15	1	-
II	minor lipids	44	10	44	13	40	4	43	8	-
III	sugars	11	3	11	5	10	6	12	5	3
IV	acids, AS	38	15	37	19	39	27	38	24	1
Σ I - IV		123	38	119	38	115	45	108	38	4
Xiushui 110 vs. Os-lpa-XS110-2										
I	FAME	30	13	33	15	27	3	26	15	-
II	minor lipids	47	8	41	30	43	9	38	27	1
III	sugars	10	5	10	1	10	2	11	3	1
IV	acids, AS	31	1	32	17	33	21	33	22	-
Σ I - IV		118	27	116	63	113	35	108	67	2

[a] Number of peaks included for comparison of a single fraction (peak height > 1000 µV)

[b] Number of peaks statistically significant different between wild-type and mutant line ($p < 0.05$)

[c] Number of peaks statistically significantly different between wild-type and mutant line at all four field trials

On average, a total of 117 peaks were included for comparison of the *indica* wild-type Xieqingzao B and the *lpa* mutant lines *Os-lpa*-XQZ-1. Assessment via Chrom*pare* demonstrated that at each field trial on average 19 peaks (16 %), were statistically significantly different ($p < 0.05$) between the wild-type and mutant. The methyl esters of myristic and pentadecanoic acid (fraction I), TMS derivatives of fructose, glucose and sucrose (fraction III) and phosphate (fraction IV) were found to be consistently different at the five field trials in 2005/2006 (Table 13). However, only phosphate

turned out to be consistently different at the two field trials in 2003/2004 (Table 12). Therefore, this compound remains as statistically significant and consistent difference between Xieqingzao B and *Os-lpa*-XQZ-1.

Table 13: Peak-based comparison of chromatograms obtained by metabolite profiling of wild-types Xieqingzao B and Xiushui 110 and mutant lines *Os-lpa*-XQZ-1, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 grown in 2005/2006.

	field trial										
	Hainan		Jiaxing		Hangzhou		Fuzhou		Guangzhou		
wild-type vs. mutant	total[a]	diff.[b]	total	diff.	total	diff.	total	diff.	total	diff.	consistent differences[c]
XQZ vs. *lpa*-XQZ-1	121	21	120	21	130	38	140	41	123	29	6
XS110 vs. *lpa*-XS110-1	144	58	121	47	118	50	135	51	111	48	5
XS110 vs. *lpa*-XS110-2	128	32	107	21	107	28	121	44	101	24	2

[a] Number of peaks included for comparison in fractions I-IV (peak height > 1000 µV)

[b] Number of peaks statistically significant different between wild-type and mutant line in fractions I-IV ($p < 0.05$)

[c] Number of peaks statistically significantly different between wild-type and mutant line at all five analyzed field trials

For the comparison of the *japonica* wild-type Xiushui 110 and the *lpa* mutant *Os-lpa*-XS110-1, on average, a total of 122 peaks were included for comparison of which 46 (38 %) were statistically significantly different at each field trial. However, as shown for the *indica* wild-type and *lpa* mutant, only a few of these peaks turned out to be statistically significantly different between Xiushui 110 and *Os-lpa*-XS110-1 at all four field trials (i.e. consistent differences) in 2003/2004. The compounds were identified as TMS derivatives of *myo*-inositol, galactose and raffinose (fraction III) and phosphate (fraction IV). These consistent differences have been confirmed for the comparison of the wild-type and *lpa* mutant grown at the five field trials in 2005/2006 (Table 13). Additionally, the methyl ester of pentadecanoic acid (fraction I) turned out to be consistently different for this growing season.

Results obtained by comparative metabolite profiling of the wild-type Xiushui 110 and mutant line *Os-lpa*-XS110-2 are shown in Tables 12 and 13. For each field trial on average 34 % of the peaks included for comparison were statistically significantly

different between wild-type and *Os-lpa*-XS110-2. However, consistent differences at all four locations in 2003/2004 were only observed for two compounds that were identified as 24-methylenecycloartanol (fraction II) and *myo*-inositol (fraction III). The TMS derivatives of phosphate and again *myo*-inositol turned out to be statistically significantly and consistently different at the five field trials in 2005/2006. Semi-quantifications (comparison of detector responses) of phosphate for the *indica* and *japonica lpa* rice mutants are shown in Figure 28.

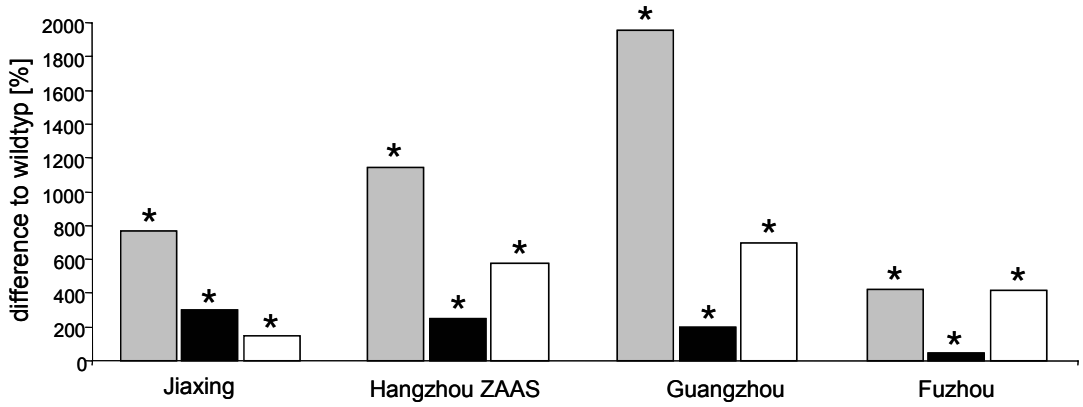

Figure 28: Mean standardized peak heights (triplicate analysis) of phosphate between wild-types Xiushui 110 and Xieqingzao B and low phytic acid mutants *Os-lpa*-XS110-1 (■), *Os-lpa*-XS110-2 (■) and *Os-lpa*-XQZ-1 (□) grown at four field trials in 2005. An asterisk indicates a statistically significant difference from the wild-type ($p < 0.05$).

The levels of phosphate were statistically significantly increased (except for *Os-lpa*-XS110-2 grown at Hainan 2003/2004) compared to the wild-types in all three mutants at all analyzed field trials. The increase was much more pronounced in *Os-lpa*-XS110-1 (+270 to +1900 %) than in *Os-lpa*-XQZ-1 (+160 to 700 %) and *Os-lpa*-XS110-2 (+150 % to +337 %).

The increase of the phosphate content in the three mutants observed in all field trials is a change expected from the effect intended by the mutation. Other phosphorylated metabolites, which might be anticipated from the influence of the significant increase in phosphate on cell metabolism, were not detected. However, it has to be kept in mind, that only low molecular weight compounds are covered by the used method. The increase in inorganic phosphate is typically used as a marker for the selection of *lpa* mutant lines [92, 94, 98]. It has been shown that despite a block in phytic acid

biosynthesis seed total phosphorus in *lpa* mutants is not decreased due to increased levels of inorganic phosphate [180].

The TMS derivative of *myo*-inositol was shown to be statistically significantly and consistently different between the two *japonica lpa* mutants *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 and the wild-type Xiushui 110. The two *lpa* rice mutants exhibited characteristic quantitative patterns of *myo*-inositol. The level of *myo*-inositol was significantly increased in *Os-lpa*-XS110-1 compared to the wild-type, whereas *Os-lpa*-XS110-2 exhibited consistently decreased levels compared to Xiushui 110. Contents of *myo*-inositol and additionally raffinose and galactose, which had been significantly and consistently increased in *Os-lpa*-XS110-1 compared to the wild-type, are exemplarily shown for the field trials in 2003/2004 in Table 14. For raffinose decreases in contents in *Os-lpa*-XS110-2 were also observed at all locations; however, for this compound the difference compared to the wild-type was statistically significant ($p < 0.05$) only at one field trial (Hainan).

Table 14: Contents of *myo*-inositol, raffinose, galactose, and galactinol in wild-type Xiushui 110 and mutant lines (*Os-lpa*-XS110-1, *Os-lpa*-XS110-2) at four field trials.

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

[a] mean ± standard deviation (n = 3)

[b] below limit of quantification (4 µg/g)

[*] statistically significantly different compared to wild-type ($p < 0.05$)

4.2.4.2 Link between mutation events and metabolic changes

The metabolites *myo*-inositol, raffinose and galactose are metabolites linked to the biogenetic pathways leading to phytic acid. The first step in the biosynthesis of phytic acid is the conversion of D-glucose 6-phosphate to Ins(3)P_1 catalyzed by MIPS [52, 53] followed by phosphorylation steps of *myo*-inositol monophosphate to phytic acid. In addition, free *myo*-inositol formed through dephosphorylation of Ins(3)P_1 by *myo*-inositol monophosphatase (MIP) acts as intermediate in the biosynthesis of phytic acid [59, 181]. As shown in Figure 29, the biosynthesis of raffinose is linked to the early biosynthetic pathway of phytic acid. Raffinose is synthesized by attaching galactose to sucrose involving *myo*-inositol as a carrier of galactose activated as galactinol [182, 183].

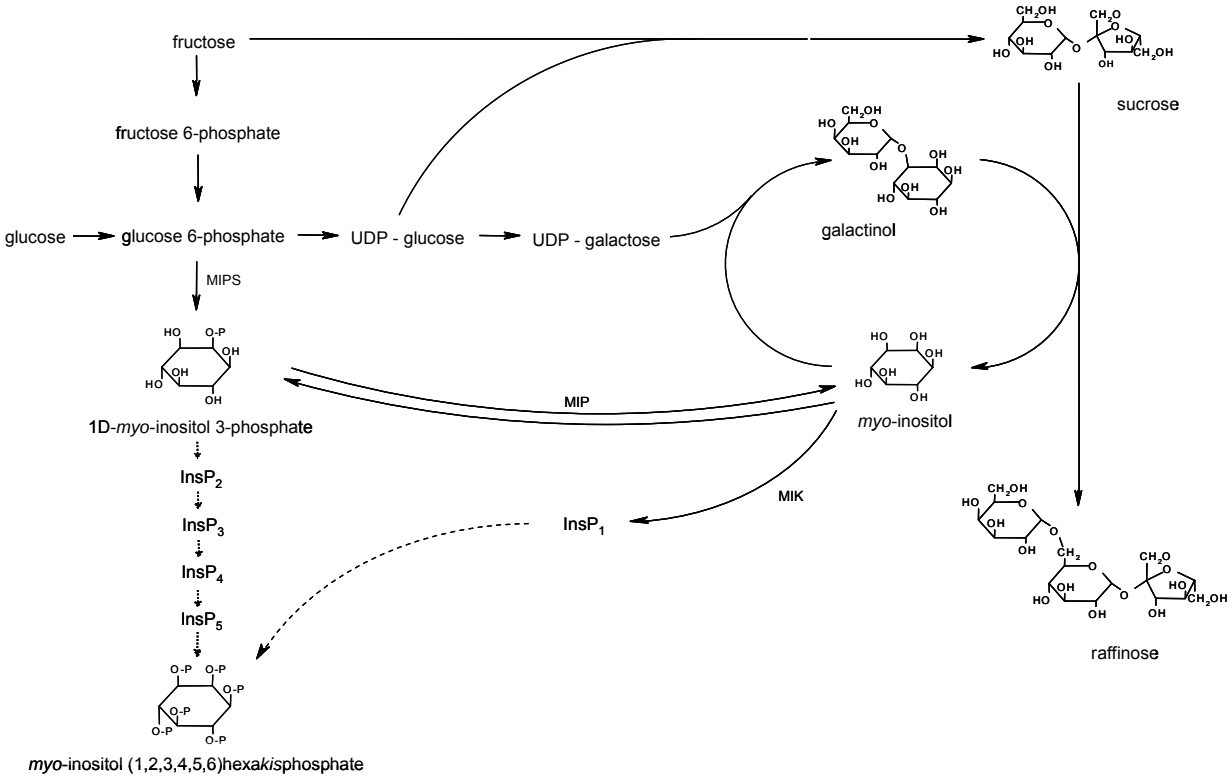

Figure 29: Plant biosynthetic pathways leading to phytic acid and raffinose. Dotted lines indicate not fully clarified pathways. MIPS, 1D-*myo*-inositol 3-phosphate synthase; MIP, *myo*-inositol monophosphatase; MIK, *myo*-inositol kinase.

The reduction of phytic acid content in the indica *lpa* mutant *Os-lpa*-XQZ-1 is accompanied by an accumulation of inorganic phosphorus. Thus, the level of total

phosphorus revealed no changes between the wild-type and the mutant [99]. An accumulation of lower inositol phosphates has not been observed. Except for decreased contents of phytic acid and increased levels of free phosphate, no further consistent metabolic changes have been observed for this compared to the wild-type Xieqingzao B. The nature of the mutation in this mutant has not been finally clarified. However, low phytic acid mutants of rice [92] and maize [94] which showed increased content of inorganic phosphorus but no accumulation of lower inositol phosphates have been classified as *lpa*-1 mutants. The phytic acid decrease in *lpa*1 maize had originally been correlated to a mutation of the MIPS gene [184]; however, it is now known that the *lpa1* mutation occurred in one of the multidrug resistance protein (MRP) genes, *ZmMRP3*, a gene encoding an ABC transporter [89]. The *lpa1* mutation is located on a chromosome different from the MIPS gene in rice [92], but anti-sense inhibition of the MIPS gene also resulted in a significant increase in inorganic phosphorus [87, 88].

The *japonica* rice mutant *Os-lpa*-XS110-1 exhibited statistically significantly increased levels of *myo*-inositol, raffinose and galactose in all field trials. In addition, the content of the metabolically related sugar galactinol (which had not been covered by the Chrom*pare* assessment due to the thresholds set for comparisons) was shown to be increased compared to the wild-type (Table 14). Similar increases in raffinose and/or *myo*-inositol levels have been observed in *lpa*-2 barley [185] and *lpa*-2 maize mutants [95]. However, in both cases the decrease in phytic acid content was accompanied by accumulation of lower inositol phosphates. *In-vitro* activity assays indicated that an inositol phosphate kinase (Zmlpk) may be involved in the late steps of the phosphorylation of inositol phosphate. Accordingly, the mutation of the Zmlpk gene resulted in an accumulation of $InsP_3$, $InsP_4$, and $InsP_5$ [95]. In the case of *Os-lpa*-XS110-1 no accumulation of $InsP_3$, $InsP_4$ or $InsP_5$ was detected.

Recently, an *lpa*3 maize mutant was discovered exhibiting increased *myo*-inositol levels without accumulation of lower inositol phosphates [59]. The gene affected by the mutation was shown to encode a *myo*-inositol kinase (MIK). This enzyme phosphorylates *myo*-inositol but neither *myo*-inositol monophosphate nor $InsP_2$ are accepted as substrates.

The metabolite profiling data obtained for *Os-lpa*-XS110-1 indicate a disturbance in this latter biosynthetic pathway of phytic acid leading to a tailback of the metabolic flux. The increased contents of *myo*-inositol, raffinose, galactose and galactinol in

combination with the absence of $InsP_3$, $InsP_4$ and $InsP_5$ lend experimental evidence to the assumption that the mutation in *Os-lpa*-XS110-1 affected early phosphorylation steps of *myo*-inositol and may therefore be similar to the *lpa*-3 mutation in maize. This metabolite profiling-based prediction is consistent with mapping results, which placed the *lpa* mutation in *Os-lpa*-XS110-1 on a site very close to the locus which encodes the putative MIK gene in rice [99].

The reduction of phytic acid content in *Os-lpa*-XS110-2 is accompanied by decreased levels of *myo*-inositol and raffinose without accumulation of lower inositol phosphates. Mutation of the MIPS gene in *lpa* soybean caused reduced phytic acid level as well as reduced levels of *myo*-inositol and raffinose [103]. In potato inhibition of $Ins(3)P_1$ synthesis by antisense insertion of the MIPS gene resulted in decreased levels of *myo*-inositol, galactinol and raffinose [166]. Therefore, reduced levels of *myo*-inositol and raffinose in *Os-lpa*-XS110-2 may indicate a disturbance in this early part of the biosynthesis of phytic acid. Taking also into account the absence of lower inositol phosphates, *Os-lpa*-XS110-2 may be similar to an *lpa*-1 maize mutant. Preliminary results of ongoing molecular genetic analyses are in agreement with this metabolite-based prediction. However, a final confirmation is still pending.

4.2.4.4 Biological variability of rice metabolites

The numbers of statistically significant differences ($p < 0.05$) in metabolites between wild-type rice and the mutants at each field trial (on average 16 % for *Os-lpa*-XQZ-1, 38 % for *Os-lpa*-XS110-1 and 34 % for *Os-lpa*-XS110-2) are in the same orders of magnitude as those determined for low phytic acid maize mutants. Application of a GC/MS metabolite profiling approach similar to the one employed in this study revealed 10 % of the detected metabolites to be statistically significantly different ($p < 0.05$) between wild-type maize and an *lpa*-1 mutant and 29 % to be different between wild-type maize and an *lpa*-3 mutant [186]. It is also interesting to note that GC-MS metabolite profiling of potatoes revealed on average 50 % of the polar metabolites analyzed to be statistically significantly different between tubers of transgenic lines altered in sucrose catabolism and wild-type controls grown in parallel in a greenhouse [11]. Comparable studies on transgenic rice demonstrated 22 % of the metabolites analyzed to be statistically significantly different between insect-resistant Bt-rice and the isogenic control grown in the field under identical conditions;

however, only five differences turned out to be consistent when comparing datasets from two field trials [187].

In the present study the assessment of statistically significant differences observed between wild-type rice and *lpa* mutants for consistency revealed that the vast majority of differences is related to biological variability rather than to the mutation event and that only a few metabolites remained as consistently different at all growing locations. However, investigation of GC metabolite profiling data by means of a principal component analysis allowed a distinct separation of the *japonica lpa* mutant *Os-lpa*-XS110-1 grown at five locations in 2005/2006 from the corresponding wild-type Xiushui 110 indicating a more pronounced influence of the mutation rather than a location-dependent impact (Figure 30). In contrast to the clear separation of *Os-lpa*-XS110-1, the separation of *Os-lpa*-XS110-2 from the wild-type was less pronounced. However, an obvious differentiation of the rice samples according to their growing locations was still observed. As shown in Figure 30, samples grown at different locations, e.g. Hainan and Jiaxing were clearly separated from each other which confirm the influence of the environment-related biological variability of the metabolite spectrum in the rice wild-types and the *lpa* mutants.

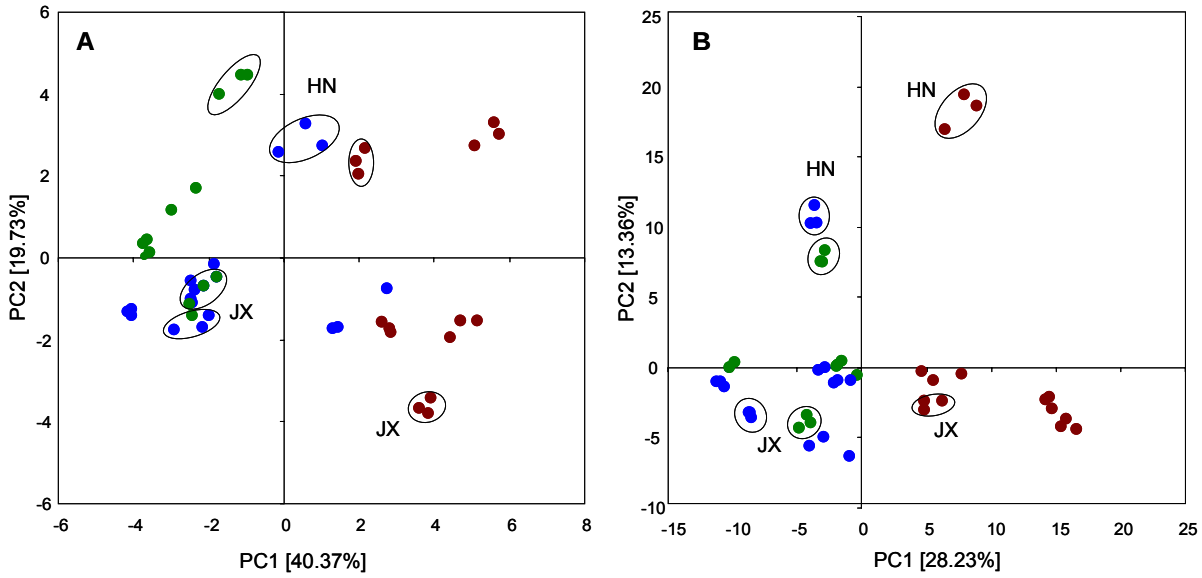

Figure 30: Principal component analysis of standardized GC-FID metabolite profiling data from the polar fractions III (A) and IV (B) of the *japonica* wild-type rice Xiushui 110 (●) and the low phytic acid mutants *Os-lpa*-XS110-1 (●) and *Os-lpa*-XS110-2 (●) grown at five field trials in 2005/2006; marked field trials: HN, Hainan; JX, Jiaxing.

In order to assess the observed statistically significantly differences between the rice wild-types and low phytic acid mutants, a conventional brown rice metabolite profiling database was established containing the two varieties *indica* and *japonica* (*n*=38), red and black rice (*n*=15) and various commercial samples from Italy, Spain, India and the USA (*n*=8). These samples were subjected to the metabolite profiling procedure as described in 3.2.3. Finally, the database contained 732 standardized chromatograms (including four non-polar and polar fractions, *n*=3) and a total of nearly 90 000 data points. Multivariate and univariate analyses were used to evaluate the differences between the mutants and wild-types in the light of a natural variability of a broad spectrum of conventional brown rice samples.

For the assessment of the total variance between Xieqingzao B and *Os-lpa*-XQZ-1 and Xiushui 110 and *Os-lpa*-XS110-1 / *Os-lpa*-XS110-2, the wild-types and *lpa* mutants were subjected together with the samples from the conventional rice database to a principal component analysis (Figure 31). The PCA plots of fractions I – IV resulted in visually distinguishable groups between the metabolite profiling data from wild-types, *lpa* mutants and conventional rice samples. However, the overall variance of the different conventional rice samples, characterized by different genetic backgrounds and growing locations, is still more pronounced than the variance between the low phytic acid mutants and the corresponding wild-types.

In addition to the multivariate approach, statistically significant differences between wild-types and *lpa* mutants were assessed in the light of natural variability following a univariate approach by means of Chrom*pare*. Therefore, the metabolite profiles of the *lpa* mutants were compared to those of the established conventional rice database. Table 15 exemplarily shows the peak-based comparison between *Os-lpa*-XS110-1 and Xiushui 110 grown at Jiaxing in 2005. The automated Chrom*pare* analysis revealed a total of 47 statistically significantly different peaks between wild-type and *lpa* mutant. However, a comparison of the peaks from the *lpa* mutant with the minimum / maximum peak heights of the conventional database revealed only two remaining different peaks in fraction IV which exceed the maximum peak height in the conventional database. One of these two compounds was identified as glutamine.

In order to evaluate the peak magnitudes for the compounds found to be statistically significantly different in a *lpa* mutant compared to the wild-type, the factor f_R for each peak was calculated (Equation 2).

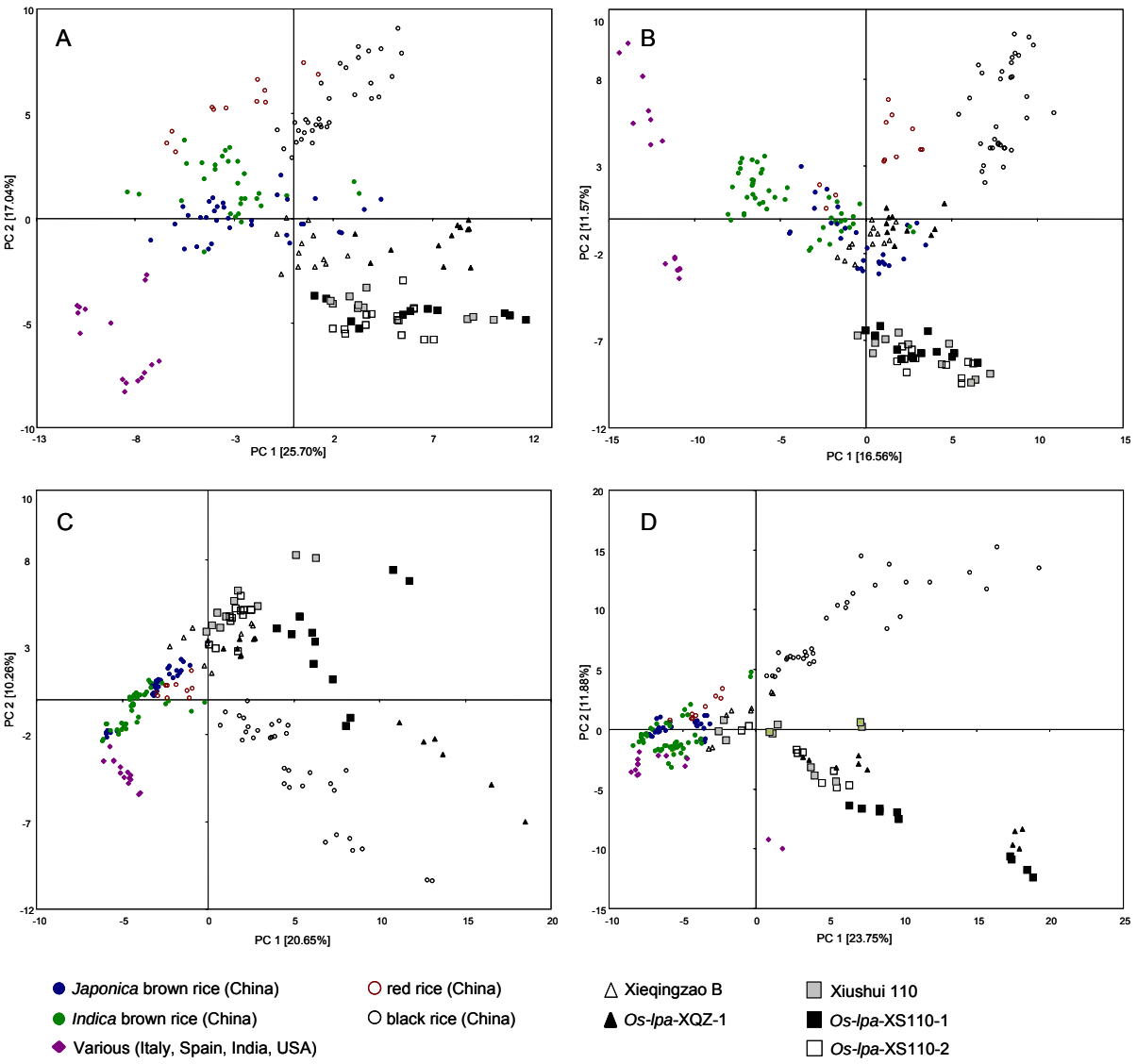

Figure 31: Principal component analysis of standardized GC-FID metabolite profiling data from fraction I (A), II (B), III (C) and IV (D) of conventional brown rice, low phytic acid rice mutants and corresponding wild-types.

Equation 2:
$$f_R = \frac{mean_{Sample} - \min_{Database}}{\max_{Database} - \min_{Database}}$$

This factor describes the relative position of a standardized peak height (mean, *n* = 3) in a single rice sample, e.g. *Os-lpa*-XS110-1 within the variation limit (minimum / maximum peak heights) of the conventional database (Table 15).

Table 15: Comparative analysis of the *lpa* rice mutant *Os-lpa*-XS110-1 grown 2005 at Jiaxing, of the corresponding wild-type and of conventional rice samples

fraction	compound[b]	statistically significant differences [%][a]		f_R[d]
		to Xiushui 110	to the minimum / maximum of the database[c]	
I	C15:0	+55	-	0.44
	C17:0	+8	-	0.57
	C20:1	-20	-	0.09
	C20:0	-21	-	0.27
	C22:0	-25	-	0.25
	C24:0	-11	-	0.39
	C26:0	-29	-	0.23
II	C18:0-OH	+105	-	0.21
	C23:0	-96	-	0.06
	n.i.[e]	-20	-	0.42
	campesterol	-16	-	0.78
	Δ^7-campestenol	-46	-	0.25
	n.i.	-31	-	0.21
	Δ^5-avenasterol	-28	-	0.15
	n.i.	+37	-	0.74
	n.i.	-37	-	0.31
	cycloartenol	-23	-	0.60
	Δ^7-avenasterol	-13	-	0.32
	24-methylenecycloartanol	+32	-	0.22
	C34:0-OH	-31	-	0.16
III	glycerol	+52	-	0.07
	fructose	+287	-	0.13
	fructose	+286	-	0.11
	fructose	+131	-	0.07
	glucose	+37	-	0.13
	galactose	+131	-	0.53
	glucose	+57	-	0.15
	myo-inositol	+124	-	0.59
	raffinose	+81	-	0.41
IV	valine	+151	-	0.57
	n.i.	+355	-	0.12
	phosphate	+764	-	0.40
	isoleucine	+106	-	0.38
	proline	+123	-	0.29
	n.i.	+231	-	0.32
	fumaric acid	+98	-	0.29
	serine	+106	-	0.69
	threonine	+184	-	0.28
	malic acid	-584	-	0.01
	aspartic acid	+41	-	0.78
	n.i.	+391	-	0.38
	glutamine	+380	+129	2.29
	n.i.	+339	+44	1.44
	n.i.	+103	-	0.18
	citric acid	-900	-	0.02
	n.i.	+56	-	0.52
	tryptophane	+146	-	0.65

[a] error probability: 5%

[b] Compounds in fraction I identified as fatty acid methyl esters, in fractions II to IV as trimethylsilylated derivatives

[c] Database containing 61 brown rice samples from China, Italy, Spain, India and USA

[d] see Equation 1

[e] n.i., not identified

For the assessment of the natural variability of metabolites, which were found to be statistically significantly and consistently different between the *lpa* mutants and the corresponding *indica* and *japonica* wild-types, Figure 32 shows the standardized peak heights of phosphate, *myo*-inositol and raffinose in the *lpa* mutants and the set of conventional rice samples.

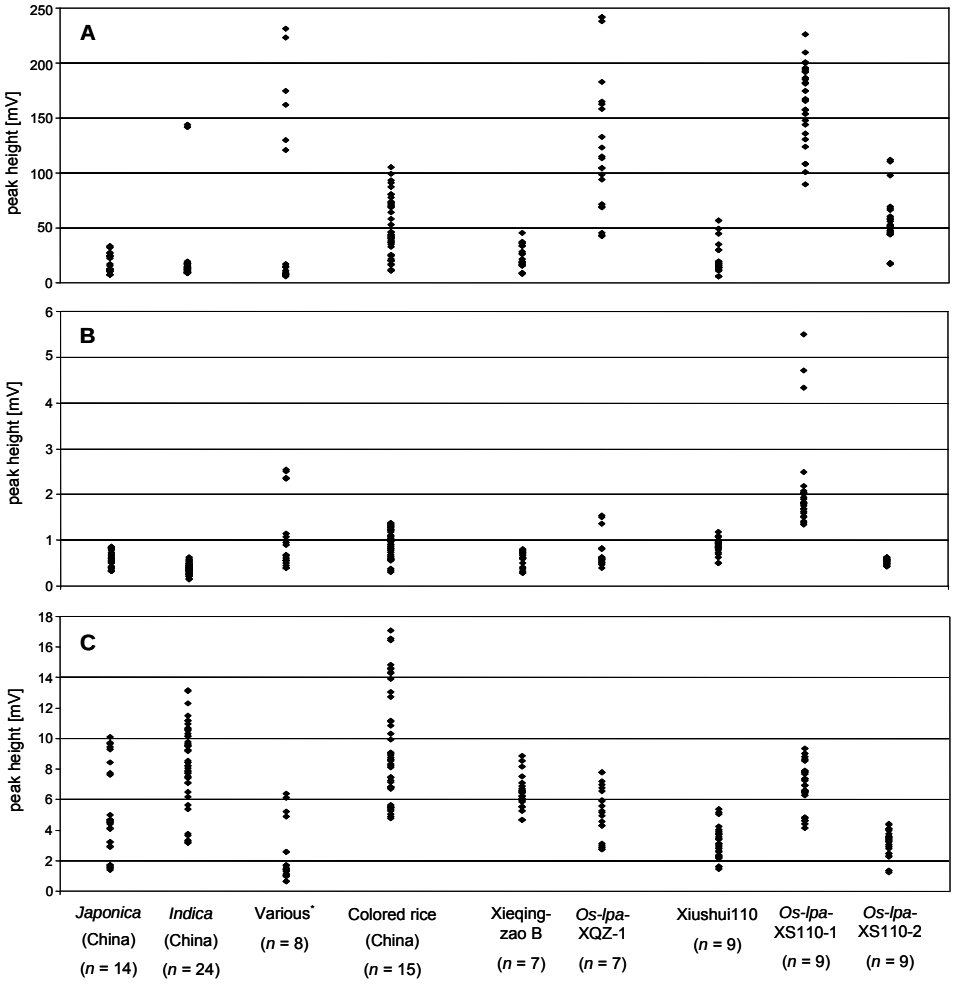

Figure 32: Standardized peaks heights of TMS derivatives of phosphate (A), *myo*-inositol (B) and raffinose (C) in conventional brown rice, low phytic acid mutants and corresponding wild-types. * Containing brown rice samples from Italy (*n*=4), Spain (*n*=2), India (*n*=1) and USA (*n*=1).

Except for the *lpa* mutant *Os-lpa*-XS110-1 grown at Hainan in 2005/2006, levels of *myo*-inositol in the *indica* and *japonica* wild-types and *lpa* mutants were within the natural range determined for the conventional brown rice samples. For phosphate and raffinose, both, wild-types and *lpa* mutants were within the natural ranges of the conventional rice database.

4.2.5 Conclusions

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

To address the human need of an adequate mineral absorption, especially in developing countries where people rely on phytate-rich staple foods such as rice, generation of low phytic acid mutants may help to improve mineral deficiencies by increasing the mineral bioavailability in such food. However, it should be considered that the total mineral absorption from food depends not only on decreased phytic acid contents in raw material but also on other food constituents or factors such as food processing. Moreover, it has to be kept in mind that *lpa* mutants might have an altered growing performance compared to wild-type crops.

4.3 INVESTIGATION OF LOW PHYTIC ACID SOYBEAN MUTANTS

4.3.1 Introduction

During the past years, various crops, e.g. rice, maize, barley and wheat with lowered contents of phytic acid have been developed [61]. For soybean, low phytic acid (*lpa*) crops have been produced by genetic engineering [89, 188] and by mutation breeding through chemical mutagenesis [102, 103] and γ-irradiation [93].

Different mutation targets have been shown to be responsible for the *lpa* phenotype in soybean mutants [189]. A single base change in the *myo*-inositol 1-phosphate synthase (MIPS1) gene resulted in a -50 % decreased phytic acid content in LR33 accompanied by a nearly molar equivalent increased level of inorganic phosphorus (P_i) compared to the wild-type [103]. Two genes (*pha*1 and *pha*2) have been identified as mutation targets in the *lpa* soybean mutant M153 (CX1834) [190, 191]. Similar to LR33, this mutant showed a molar equivalent increase in P_i while the phytic acid content was decreased by -55 % [102].

Low phytic acid crop mutants are typically selected and classified on the basis of their altered levels of P_i. In addition to altered levels of phytic acid and P_i, the induced mutations were shown to result in further metabolic changes in these crops. For LR33 soybeans, targeted analysis revealed decreased levels of *myo*-inositol, raffinose and stachyose, whereas the sucrose content was increased [103]. For high P_i lines derived from M153 soybeans, levels of sucrose, raffinose and stachyose have been reported to be unchanged compared to the parental line [191].

Metabolite profiling techniques aspires to provide a comprehensive picture of the metabolites present in a biological system. The metabolite profiling study on novel *lpa* rice mutants described in 4.2 demonstrated the applicability of this approach to detect metabolic differences between the *lpa* mutants and the corresponding wild-type. Consideration of these differences in the light of the pathways involved in the biosynthesis of phytic acid allowed an assumption on the mutation targets in these mutants which were shown to be in agreement with molecular mapping results [99].

The study objects of this investigation were the two novel *lpa* soybean mutants *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2 developed by γ-irradiation [93]. The phytic acid phosphorus reduction of >60 % in *Gm-lpa*-TW75-1 was shown to result from a two

base pair deletion in the MIPS1 gene. However, the mutation locus was shown to be independent from that in LR33. Similar to LR33 and M153, this *lpa* soybean mutant exhibited significantly increased P_i levels. In contrast to previously described *lpa* soybean mutants, the phytic acid reduction in *Gm-lpa*-ZC-2 is accompanied by an accumulation of lower inositol phosphates [93]. However, the genetic background of this mutation has not been fully clarified. The aim of the present study was to compare the metabolic profiles of these two *lpa* soybean mutants to the corresponding wild-types by means of metabolite profiling in order to determine the metabolic changes in the mutants and thus to confirm the suitability of metabolite profiling to assist in the elucidation of mutation events. To assess the environmental impact on the metabolite levels of the soybean wild-types and mutants were grown at different field trials and the resulting metabolite profiles were subjected to multivariate and univariate statistical analysis.

4.3.2 Analysis of inositol phosphates

The mean contents and distribution of phytic acid and lower inositol phosphates ($InsP_3$, $InsP_4$, $InsP_5$) for the wild-types (Taiwan 75, Zhechun no. 3) and the *lpa* mutants (*Gm-lpa*-TW75-1, *Gm-lpa*-ZC-2) are shown in Figure 33. The *lpa* mutants *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2 were grown side by side with their corresponding wild-types at three and four locations, respectively. Compared to wild-types, the *lpa* mutants exhibited significantly reduced phytic acid contents ranging from -42 to -66 % for *Gm-lpa*-TW75-1 and from -22 to -57 % for *Gm-lpa*-ZC-2, respectively. On average, the degree of phytic acid reduction was slightly higher for *Gm-lpa*-TW75-1 (-53 %) than for *Gm-lpa*-ZC-2 (-46 %) compared to the wild-type. For *Gm-lpa*-TW75-1, no accumulation of lower inositol phosphates was observed (Figure 33A), whereas *Gm-lpa*-ZC-2 exhibited significantly increased contents of $InsP_3$, $InsP_4$ and $InsP_5$ (Figure 33B). This is in agreement with results obtained for this mutant in a previous field trial [93]. The sum of the inositol phosphates $InsP_3$ – $InsP_6$ appears to be slightly higher in the mutant than in the wild-type (Figure 33B). However, this might be due to analytical shortcomings. It has to be kept in mind that the determination of lower inositol phosphates was based on a calibration curve of phytate only using correction factors for the lower InsP as described in literature [108].

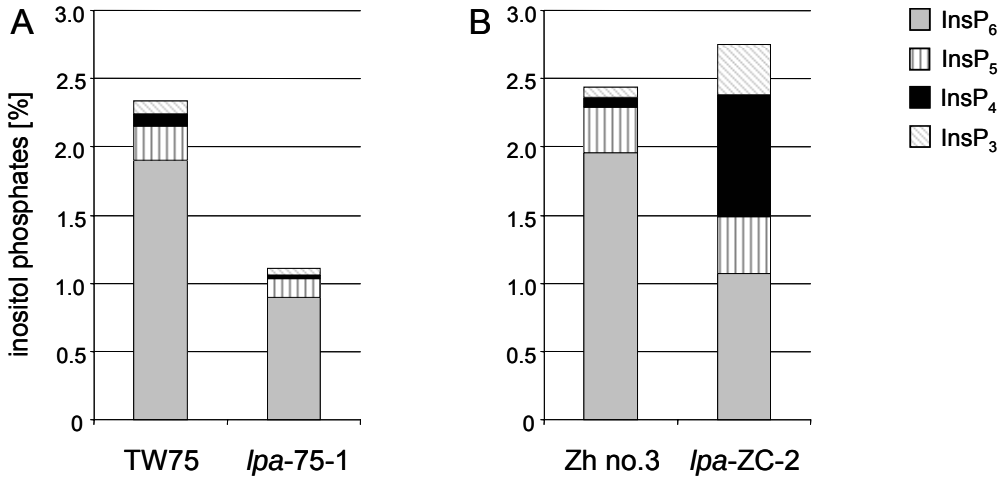

Figure 33: Mean contents of phytic acid ($InsP_6$) and lower inositol phosphates ($InsP_3$, $InsP_4$, $InsP_5$) in Taiwan 75 (TW75) and *Gm-lpa*-TW75-1 (*lpa*-75-1) (A) and Zhechun no. 3 (Zh no.3) and *Gm-lpa*-ZC-2 (*lpa*-ZC-2) (B) grown at three and four field trials, respectively.

Contents of phytic acid in the soybean wild-types Taiwan 75 and Zhechun no. 3 were within the reported natural range [192, 193].

4.3.3 Contents of divalent cations

Contents of calcium, iron, zinc and cadmium in the two *lpa* soybean mutants and their wild-types are shown in Table 16. On average, the levels of calcium in *Gm-lpa*-TW75-1 (3.04 g/kg) were increased compared to the wild-type (2.79 g/kg). However, for one of the three field experiments (Hangzhou, autumn 2005), the results were not statistically significant. No consistently pronounced differences were observed for the contents of iron and zinc. In addition, no consistent changes for the concentrations of calcium, iron and zinc were detected for the *lpa* soybean mutant *Gm-lpa*-ZC-2 compared to wild-type.

The two *lpa* soybean mutants *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2 are characterized by absence and by accumulation of lower inositol phosphates, respectively. Considering the nearly similar seed mineral levels in the two *lpa* mutants and wild-types, there is no evidence that the two mutation types differ in their impact on the mineral contents. In particular, the accumulation of lower inositol phosphates does

not seem to alter the mineral levels in the *lpa* soybean mutant compared to the wild-type.

Table 16: Contents of calcium, iron, zinc and cadmium in the low phytic acid (*lpa*) soybean mutants *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2 and the corresponding wild-types Taiwan 75 and Zhechun no. 3 (mean ± confidence interval, $n = 3$, $p < 0.05$)

growing location / season	Taiwan 75	*Gm-lpa*-TW75-1	Zhechun no. 3	*Gm-lpa*-ZC-2
	calcium [mg/kg]			
Hangzhou, spring 2005	3732 ± 51	4052 ± 45	2550 ± 30	2763 ± 40
Hangzhou, autumn 2005	2220 ± 30	2262 ± 12	2282 ± 17	1928 ± 17
Hainan, winter 2003/04	-	-	1778 ± 7	1953 ± 9
Hainan, winter 2005/06	2403 ± 7	2802 ± 57	1872 ± 7	1972 ± 17
	iron [mg/kg]			
Hangzhou, spring 2005	101 ± 1	96 ± 1	116 ± 1	127 ± 1
Hangzhou, autumn 2005	98 ± 4	91 ± 1	108 ± 1	119 ± 2
Hainan, winter 2003/04	-	-	101 ± 5	90 ± 1
Hainan, winter 2005/06	79 ± 1	78 ± 3	83 ± 1	90 ± 1
	zinc [mg/kg]			
Hangzhou, spring 2005	58 ± 1	63 ± 1	58 ± 1	59 ± 1
Hangzhou, autumn 2005	58 ± 2	56 ± 1	57 ± 4	63 ± 1
Hainan, winter 2003/04	-	-	70 ± 1	69 ± 1
Hainan, winter 2005/06	87 ± 1	92 ± 2	84 ± 1	78 ± 1
	cadmium [µg/kg]			
Hangzhou, spring 2005	183 ± 1	158 ± 4	178 ± 6	200 ± 2
Hangzhou, autumn 2005	118 ± 4	126 ± 7	110 ± 2	155 ± 3
Hainan, winter 2003/04	-	-	139 ± 6	133 ± 4
Hainan, winter 2005/06	68 ± 4	67 ± 3	62 ± 1	67 ± 2

Regarding the natural variability of mineral concentrations, both, the mean contents of calcium, iron and zinc in the soybean wild-types as well as the contents in the mutants are within the reported natural range [171, 192].

The cadmium contents of the soybean wild-types and *lpa* mutants are shown in Table 16. Only at one of three field trials (Hangzhou, spring 2005) the cadmium concentration in *Gm-lpa*-TW75-1 was found to be statistically significantly lowered

compared to wild-type. *Gm-lpa-ZC-2* exhibited predominantly increased cadmium contents (average: +13 %). The ability of lower inositol phosphates to bind cadmium has been demonstrated [194]. Therefore, the increased cadmium contents might result from the significant accumulation of lower inositol phosphates compared to the wild-type. However, this effect would have to be confirmed in further studies.

As described in 4.2.3.3, the molar ratios of phytic acid and minerals can be used to predict the relative bioavailability of minerals in humans. The phytate / mineral molar ratios of soybean wild-types and *lpa* mutants grown at Hangzhou in 2005 are shown in Table 17.

Table 17: Phytate / mineral molar ratios of *lpa* soybean *Gm-lpa*-TW75-1 and *Gm-lpa* ZC-2 and corresponding wild-types at two field-trials (Hangzhou) in 2005 (mean, *n* = 2)

	phytate / calcium[a]	phytate / iron[a]	phytate / zinc[a]
Taiwan 75	0.58	22.4	44.9
Gm-lpa-TW75-1	0.25	10.9	20.0
Zhechun no. 3	0.59	17.7	40.3
Gm-lpa-ZC-2	0.75	18.9	44.6

[a] Phytate / calcium, phytate / iron and phytate / zinc molar ratios were calculated on the basis of the sum of all detected inositol phosphates

Compared to the wild-type, the *lpa* mutant *Gm-lpa*-TW75-1 revealed significantly decreased phytate / minerals molar ratios on the basis of total inositol phosphates. However, only the calcium availability in terms of the phytate / calcium molar ratio was within the desirable range [175]. Molar ratios of phytate / iron and phytate / zinc were below the values reported for soybean powder in literature [178]. Despite improved molar ratios, the absorption of iron and zinc might be still impaired in the *lpa* mutant. As a result of the significant accumulation of lower inositol phosphates in *Gm-lpa*-ZC-2, the *lpa* mutant even exhibited increased molar ratios compared to the wild-type Zhechun no. 3.

Compared to rice, the soybean wild-types and *lpa* mutants exhibited noteworthy amounts of lower inositol phosphates. The impact of lower inositol phosphates on the inhibition of mineral absorption is controversially discussed. In earlier studies it was

reported that isolated lower inositol phosphates ($InsP_3$ and $InsP_4$) may have no or only small impact on the inhibition of calcium, iron and zinc compared to inositol pentaphosphate and phytic acid [195-197]. However, it should be considered that the results obtained from pure isolated inositol phosphates cannot simply be transferred to conventional food comprising complex mixtures of inositol phosphates. For example, $InsP_3$ and $InsP_4$ have been shown to contribute significantly to an inhibition of iron and zinc absorption in humans when eating bread [197, 198]. With respect to the not well known inhibitory effects of $InsP_3$ and $InsP_4$ in a complex food matrix, further studies would be necessary to investigate the impact of lower inositol phosphates on the mineral absorption.

4.3.4 Metabolite profiling

Freeze-dried soybean flour was subjected to a metabolite profiling procedure that resulted in four fractions containing fatty acid methyl esters and hydrocarbons (fraction I), free fatty acids, fatty alcohols and sterols (fraction II), sugars and sugar alcohols (fraction III) and amino acids, organic and inorganic acids and amines (fraction IV). The procedure is in accordance with the methodology used for rice and barley. The adaptation of the extraction and fractionation protocol to the soybean matrix required slight modifications. The higher contents of total lipids and polar compounds in soybeans [159] was taken into account by decreasing the amount of freeze-dried flour subjected to the initial extraction and by adjusting the amount of polar extract used for fractionation. The fractions were analyzed by capillary gas chromatography. GC-FID analysis resulted in the detection of a total of 613 distinct peaks in the four fractions of the two *lpa* mutants and wild-types.

4.3.4.1 Multivariate analysis

The standardized and consolidated GC data were subjected to a principal component analysis (PCA) to evaluate a potential differentiation of the soybean samples due to the parameters cultivar, type of mutation and growing location / season. Scores plots obtained for the combined fractions I-IV and for each single fraction are shown in Figure 34. Metabolic changes between the soybean samples are reflected by shifts of the scores for the first two principal components PC1 and PC2. For the scores plots

in the combined fractions I-IV, the first two PC explained 37 % of the total variance (Figure 34A). There are differentiations according to the growing locations / seasons for the wild-types as well as for the corresponding mutants. The mutant *Gm-lpa*-TW75-1 is consistently separated from the wild-type Taiwan 75 at all field trials. In contrast, the variance between the mutant *Gm-lpa*-ZC-2 and the wild-type Zhechun no. 3 is less pronounced, resulting in a clear separation at only one field trial.

The same phenomenon is reflected by the scores plots of the single fractions III (Figure 34D) and IV (Figure 34E). The distinct and consistent separations of *Gm-lpa*-TW75-1 and the wild-type Taiwan 75 indicate a strong influence of the mutation on the polar metabolite profiles in this mutant. In contrast to the polar fractions, no major shift was observed in the non-polar fractions (Figures 34B and 34C) for this mutant compared to wild-type. Instead, a location-dependent separation of the side-by-side grown mutant *Gm-lpa*-TW75-1 and the corresponding wild-type was observed which indicates a more pronounced environmental impact rather than mutational effects on the lipid profile.

Assessment of the scores plots of polar and non-polar fractions for *Gm-lpa*-ZC-2 and Zhechun no. 3 exhibited no major differences between the *lpa* mutant and the corresponding wild-type grown at one location. However, similar to the observations made for *Gm-lpa*-TW75-1 in the non-polar fractions, a considerable influence of the growing location was detected for both non-polar and polar fractions. In addition to an influence of the growing location, a significant effect was also observed for the different growing seasons. *Lpa* mutants and corresponding wild-types grown in the spring season 2005 at Hangzhou were separated from the rest of the samples in the two non-polar fractions (Figures 34B and 34C).

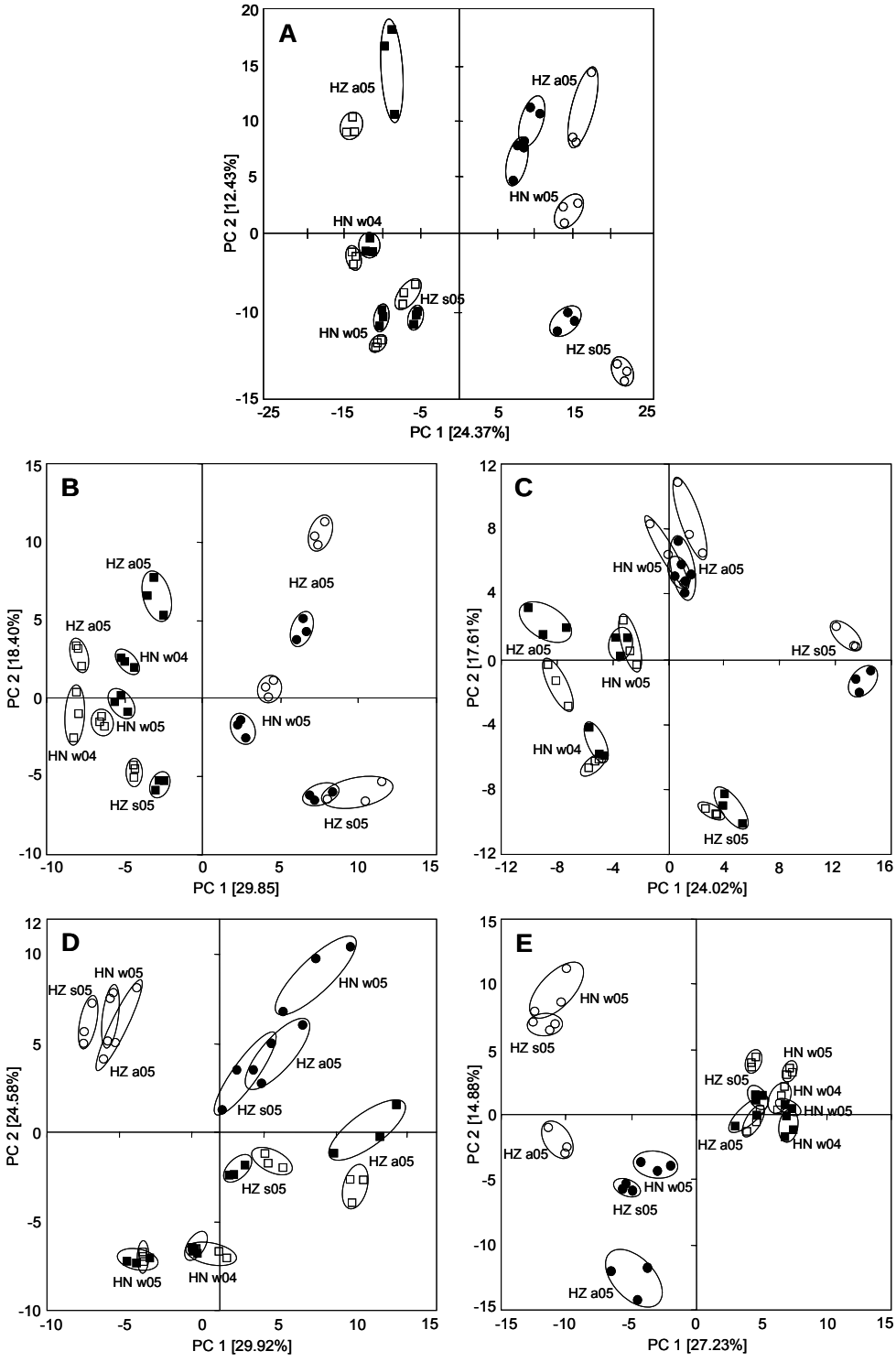

Figure 34: Principal component analyses (PCA) of standardized GC-FID metabolite profiling data from the combined fractions I – IV (A) and single fractions I (B), II (C), III (D) and IV (E) of the wild-types soybean Taiwan 75 (●) and Zhechun no. 3 (■) and *lpa* mutants *Gm-lpa*-TW75-1 (○) and *Gm-lpa*-ZC-2 (□) grown at Hainan and Hangzhou in 2004/05 – 2005/06. HN w04 and w05: Hainan winter 2004 and 2005, HZ s05 and a05: Hangzhou spring 2005 and autumn 2005.

An explanation for this could be the subtropical climatic condition at Hangzhou during this season. It was shown, that both *lpa* mutants and wild-types had a lower seedling emergence when they were produced in spring season at this location [93]. A low phytate-related impairment on seedling emergence has also been shown for M152-derived *lpa* lines especially when the plants were grown in a subtropical environment [199, 200].

4.3.4.2 Univariate analysis

For comparison of GC-FID data, peak heights and corresponding retention times were imported to Chrom*pare* [147]. Results from the comparative analysis of fractions I-IV obtained by metabolite profiling of the *lpa* mutant lines and the corresponding wild-types are shown in Tables 18 and 19.

Table 18: Peak-based comparison of chromatograms obtained by metabolite profiling of wild-type Taiwan 75 and *lpa* mutant line *Gm-lpa*-TW75-1 grown in the three field trials Hainan, winter 05/06 (Hainan 05/06), Hangzhou, spring 2005 (Hangzhou, s05) and Hangzhou, autumn 2005 (Hangzhou, a05)

		field trial						
		Hainan 05/06		Hangzhou, s05		Hangzhou a05		
fraction	compound class	total[a]	diff.[b]	total[a]	diff.[b]	total[a]	diff.[b]	consistent differences[c]
I	FAME	45	17	45	4	47	26	1
II	minor lipids	42	15	39	1	47	16	-
III	sugars	31	18	29	20	30	18	14
IV	acids, AS	52	24	70	44	43	16	8
Σ I - IV		166	74	181	69	165	76	23

[a] Number of peaks included for comparison (peak height > 500 µV)

[b] Number of peaks statistically significant different between wild-type and mutant line ($p < 0.05$)

[c] Number of peaks statistically significantly different between wild-type and mutant line at all four field trials

On average, 171 peaks were included for the comparison of *Gm-lpa*-TW75-1 vs. Taiwan 75 and 164 for *Gm-lpa*-ZC-2 vs. Zhechun no. 3. Assessment of *lpa* mutants and wild-types by means of Chrom*pare* revealed on average for *Gm-lpa*-TW75-1 68 (equates 40 %), and for *Gm-lpa*-ZC-2 35 (21 %) statistically significantly different peaks compared to the corresponding wild-type at one field trial.

Table 19: Peak-based comparison of chromatograms obtained by metabolite profiling of wild-type Zhechun no. 3 and *lpa* mutant line *Gm-lpa*-ZC-2 grown in the four field trials Hainan, (winter 04/05, winter 05/06), Hangzhou, spring 2005 (Hangzhou, s05) and Hangzhou, autumn 2005 (Hangzhou, a05)

		field trial								
		Hainan, 04/05		Hainan, 05/06		Hangzhou, s05		Hangzhou a05		
fraction	compound class	total[a]	diff.[b]	total[a]	diff.[b]	total[a]	diff.[b]	total[a]	diff.[b]	consistent differences[c]
I	FAME	40	4	42	18	39	0	39	4	-
II	minor lipids	44	6	39	12	41	1	40	16	-
III	sugars	27	3	21	3	31	4	35	12	1
IV	acids, AS	55	14	62	22	46	8	53	11	2
Σ I - IV		166	27	164	55	157	13	167	43	3

[a] Number of peaks included for comparison (peak height > 500 µV)

[b] Number of peaks statistically significant different between wild-type and mutant line ($p < 0.05$)

[c] Number of peaks statistically significantly different between wild-type and mutant line at all four field trials

The percentages of statistically significant differences in metabolites between the *lpa* mutants and wild-types for each field trial are in the same order of magnitude as those determined for comparable GC-based metabolite profiling studies on *lpa* mutants of rice (chapter 4.2) and maize [186].

The assessment of statistically significantly differences between the *lpa* soybean mutants and wild-types revealed that only some of them were consistently present at all analyzed field trials. This observation is in agreement with results obtained for the *lpa* rice mutants confirming the importance of environmental impact and natural variability on plant metabolite profiles (chapter 4.2).

The differences seen in the PCA scores plots regarding the separation of wild-types and mutants were also reflected in the number of consistent differences determined. The identification of significantly and consistently different compounds between the wild-types and *lpa* mutants is given in Table 20.

Table 20: Peak responses of compounds found to be consistently significantly different between Taiwan 75 vs. *Gm-lpa*-TW75-1 and Zhechun no. 3 vs. *Gm-lpa*-ZC-2 grown at three and four field trials, respectively.

		response [mV]			
fraction	compound	Taiwan 75	*Gm-lpa*-TW75-1	ratio	identification[1]
I	heptadecene	1.4 ± 0.1	2.2 ± 0.1	1.54	B
III	glycerol	0.7 ± 0.2	1.6 ± 0.4	2.51	A
	sorbitol	0.7 ± 0.1	1.4 ± 0.1	1.92	A
	myo-inositol	6.2 ± 1.7	0.8 ± 0.1	0.12	A
	sucrose	200 ± 31	306 ± 25	1.53	A
	galactopinitol A	8.8 ± 2.9	0.7 ± 0.2	0.08	C, E
	galactopinitol B	9.2 ± 2.2	0.8 ± 0.2	0.09	C, E
	fagopyritol B1	4.1 ± 1.0	1.2 ± 0.5	0.28	C, E
	galactinol	0.7 ± 0.1	b.l.d.[2]		A
	galactose derivate	1.7 ± 0.2	0.7 ± 0.1	0.41	E
	galactose derivate	2.4 ± 0.4	0.4 ± 0.1	0.18	E
	raffinose	63 ± 5	16 ± 1	0.26	A
	ciceritol	1.9 ± 0.2	b.l.d.		D, E
	unknown[3]	1.4 ± 0.2	b.l.d.		
	stachyose	37 ± 2	0.4 ± 0.1	0.01	A
IV	phosphoric acid	37 ± 8	220 ± 44	5.93	A
	valine	1.3 ± 0.3	3.7 ± 0.6	2.88	A
	leucine	1.5 ± 0.4	4.1 ± 1.0	2.78	A
	serine	0.6 ± 0.2	1.8 ± 0.5	2.83	A
	ß-alanine	0.5 ± 0.1	1.6 ± 0.3	2.88	A
	asparagine	1.1 ± 0.2	5.7 ± 2.3	5.44	A
	citrulline	0.7 ± 0.1	2.2 ± 0.6	3.17	A
	tyrosine	1.4 ± 0.8	0.5 ± 0.1	0.34	A
		Zhechun no. 3	*Gm-lpa*-ZC-2		
III	*myo*-inositol	2.2 ± 0.3	4.3 ± 0.5	1.96	A
IV	phosphoric acid	45 ± 7	94 ± 12	2.11	A
	syringic acid	2.4 ± 0.9	3.7 ± 1.3	1.57	B

[1] Identification according to

A mass spectrometric data and retention time of reference compound

B NIST 02 MS library

C Schweizer et al., 1978 [201]; Schweizer and Horman, 1981 [202]

D Quemener and Brillouet, 1983 [203]

E MS data (see Figure 36)

[2] below limit of detection

[3] no MS data available

For *Gm-lpa*-TW75-1, 23 of them were found to be consistently different at all field trials, whereas only three were found to be consistently different for *Gm-lpa*-ZC-2 compared to the wild-type.

The three consistently different compounds in *Gm-lpa*-ZC-2 were present in fractions III and IV and were identified as trimethylsilyl (TMS) derivatives of phosphate, *myo*-inositol and syringic acid. Their levels were shown to be significantly increased compared to the wild-type. The majority of the 23 consistent differences between the *lpa* mutant *Gm-lpa*-TW75-1 and its wild-type were detected in fraction III. GC-FID chromatograms of fractions III obtained from the wild-type *Gm-lpa*-TW75-1 and the *lpa* mutant *Gm-lpa*-TW75-1 grown in Hangzhou (spring 2005) are exemplarily shown in Figure 35.

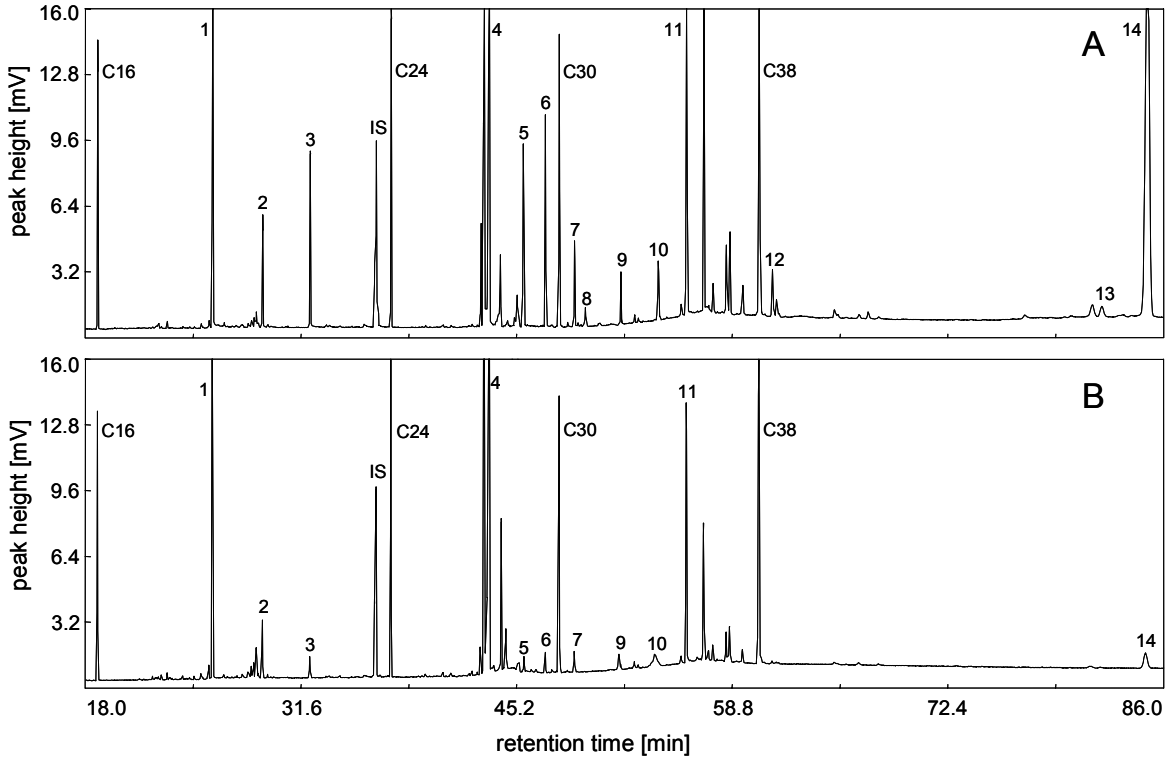

Figure 35 GC-FID chromatograms of fraction III obtained for the wild-type Taiwan 75 (A) and the *lpa* mutant *Gm-lpa*-TW75-1 (B) grown in spring season 2005 in Hangzhou. Peaks 1-14: TMS derivatives of D-pinitol (1), *chiro*-inositol (2), *myo*-inositol (3), sucrose (4), galactopinitol A (5), galactopinitol B (6), fagopyritol B1 (7), galactinol (8), non-identified galactose derivatives (9, 10), raffinose (11), ciceritol (12), unknown (13) and stachyose (14). Peaks C16 (hexadecane), C24 (tetracosane), C30 (triacontane), C38 (octatriacontane): retention time standards. IS: internal standard phenyl-ß-D-glucopyranosid.

TMS derivatives of the significantly changed compounds glycerol, sorbitol, *myo*-inositol, sucrose, galactinol, raffinose and stachyose were identified by comparison of mass spectrometric data and retention times to those of authentic reference compounds. In addition, the galactosyl cyclitols galactopinitol A, galactopinitol B, fagopyritol B1 and ciceritol were tentatively identified based on comparison of their mass spectral data (Figure 36A-D) and of their retention behaviour to those described for these galactosyl cyclitols described in literature [201-203].

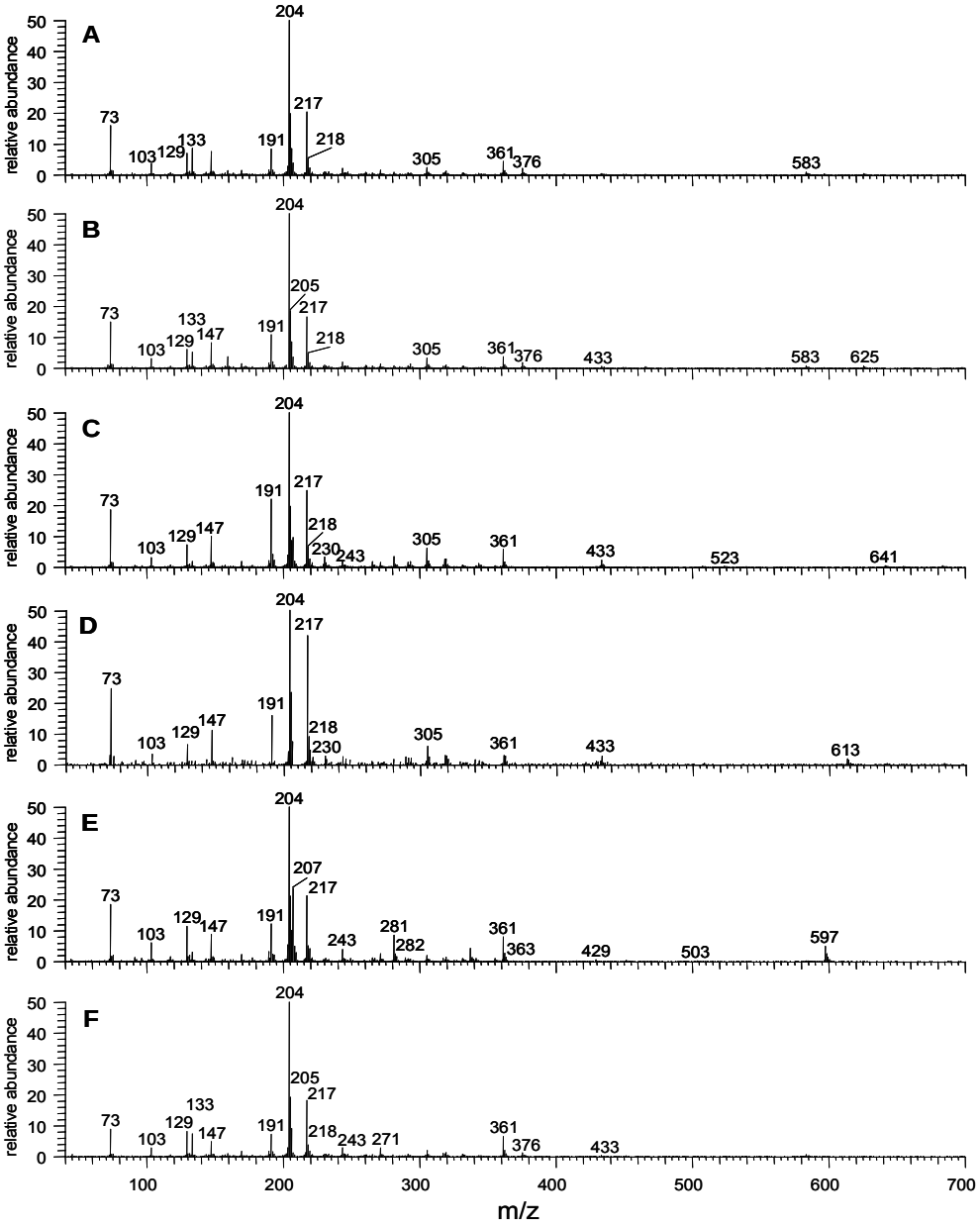

Figure 36: Electron impact mass spectra of tentatively identified TMS derivatives of galactopinitol A (A), galactopinitol B (B), fagopyritol B1 (C), ciceritol (D) and of non-identified galactose derivatives (E, F).

Three other consistently different peaks detected in fraction III could not been finally identified. However, the MS data of two of the peaks indicate that these compounds are also galactose derivatives (Figure 36E and F). In legumes, a broad range of cyclitols and galactosyl derivatives has been reported [204-207].
The mutant *Gm-lpa*-TW75-1 exhibited significantly increased levels of glycerol, sorbitol and sucrose, whereas the levels of all other compounds found to be consistently different in fraction III were shown to be statistically significantly decreased.

4.3.4.3 Biogenetic aspects

The biosynthesis of phytic acid starts with the conversion of D-glucose 6-phosphate to 1D-myo-inositol 3-phosphate ($Ins(3)P_1$) through D-*myo*-inositol 3-phosphate synthase (MIPS1) followed by kinase catalyzed phosphorylation to higher homologous [60]. In addition, *myo*-inositol formed through the dephosphorylation of $Ins(3)P_1$ is considered as an important intermediate in the pathway leading to phytic acid (Figure 2) [60, 61].

Gm-lpa-TW75-1

The *lpa* mutant *Gm-lpa*-TW75-1 is characterized by the absence of lower inositol phosphates. The reduction of phytic acid in *Gm-lpa*-TW75-1 is accompanied by a molar equivalent increase of P_i [93]. Therefore, the observed significant increase in free phosphate was expected. In addition to the altered phosphate content, the comparative metabolite profiling of *Gm-lpa*-TW75-1 and Taiwan 75 revealed significantly decreased contents of *myo*-inositol, oligosaccharides and of the tentatively identified galactosyl cyclitols in the *lpa* mutant, whereas the sucrose level was shown to be increased. As shown in Figure 37, the biosynthetic pathways of phytic acid, raffinose oligosaccharides and galactosyl cyclitols are closely linked. The changes observed for these sugars by metabolite profiling are in excellent agreement with the suggestion that the phytic acid reduction in this mutant results from a lesion in the MIPS1 gene [93].

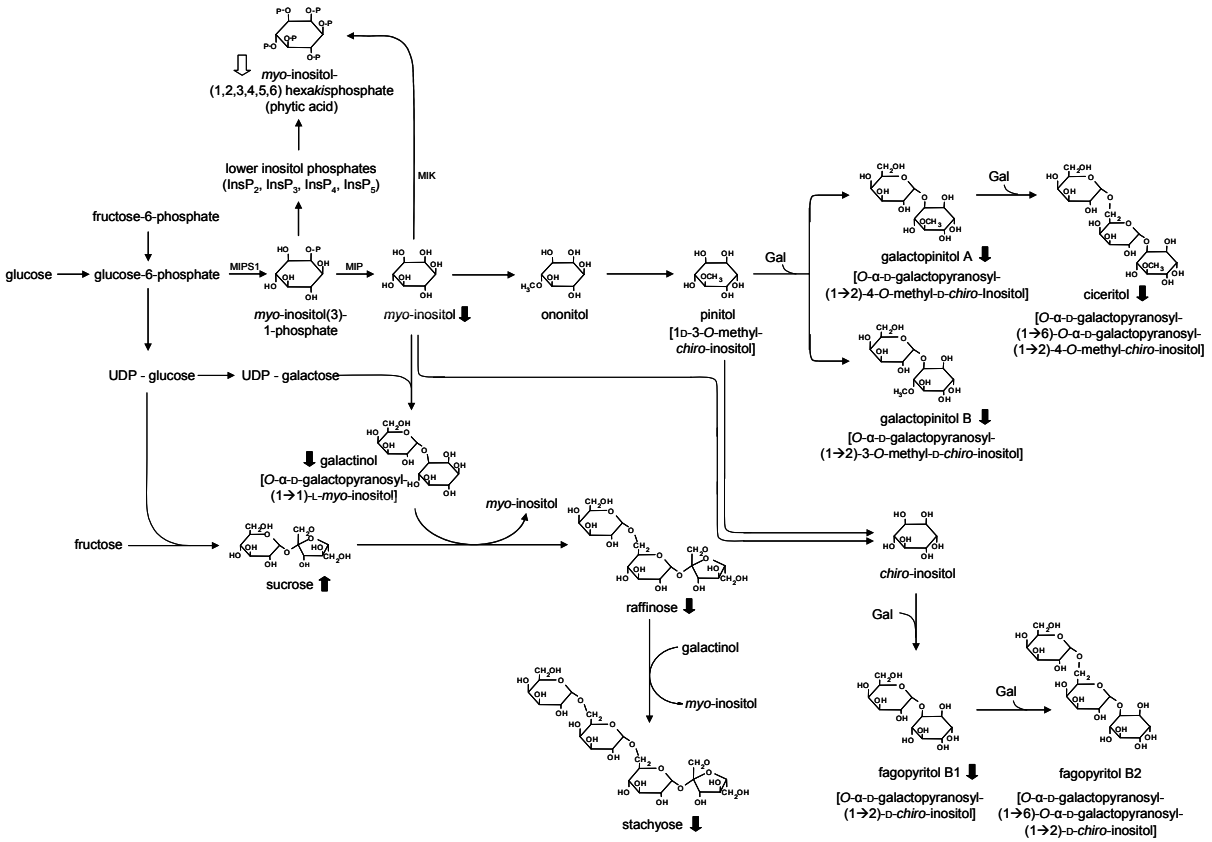

Figure 37: Link between the biosynthetic pathways leading to phytic acid, raffinose oligosaccharides and galactosyl cyclitols. MIPS1, 1D-*myo*-inositol 3-phosphate synthase; MIP, *myo*-inositol monophosphatase. Arrows indicate statistically significantly metabolic changes between the *lpa* mutant *Gm-lpa*-TW75-1 and the wild-type Taiwan 75.

Gm-lpa-ZC-2

Out of the various consistent changes observed for metabolites in *Gm-lpa*-TW75-1 compared to the wild-type, metabolite profiling of the mutant *Gm-lpa*-ZC-2 only revealed consistent increases of the contents of phosphate (the intended effect), of syringic acid and of *myo*-inositol compared to wild-type. Together with the accumulation of lower inositol phosphates these metabolite profiling data clearly show that the phytic acid reductions in the two *lpa* soybeans investigated in this study are based on different types of mutation. Increased levels of *myo*-inositol have also been reported for *lpa* mutants of maize [95] and barley [185] which also showed accumulation of lower inositol phosphates [94, 98]. For *lpa* maize, a mutation in an inositol phosphate kinase (ZmIpk) gene was shown to be responsible for the

observed phenotype [95]. The mutation target in *Gm-lpa*-ZC-2 has been located by molecular mapping [93]; however, an identification of the associated enzyme(s) is still pending. The accumulation of lower inositol phosphates support that this mutation results from a lesion in one of the inositol phosphate kinase involved in the latter steps of the biosynthesis of phytic acid.

4.3.5 Outlook

Due to the improved phytate / mineral molar ratios in the *lpa* mutant *Gm-lpa*-TW75-1, feeding or consuming diets based on these *lpa* mutant should lead to an enhanced mineral utilization compared to the wild-type material. However, it has to be considered, that the applied method for mineral determination only allows the coverage of the total cation contents. A possibility to differentiate between free seed minerals and minerals bound to phytic acid would be very valuable. In addition, the knowledge on *in vivo* interactions between lower inositol phosphates and minerals in plants, a phenomenon potentially relevant for the soybean mutant *Gm-lpa*-ZC2, is still limited.

Metabolite profiling and inositol phosphate analysis of the two *lpa* soybean mutants revealed distinct differences between *Gm-lpa*-TW75-1 and *Gm-lpa*-ZC-2. The majority of statistically significantly differences between *lpa* mutants and wild-types were shown to be related to environmental impact and natural variability rather than to the mutation event. The consistent metabolic changes observed in *Gm-lpa*-TW75-1 confirmed the suggested mutation target [93] and demonstrate the power of metabolite profiling in assisting in the elucidation of mutation events.

The unbiased and non-targeted screening of a broad spectrum of metabolites also increases the probability to detect effects not intended by the irradiation process and thus may contribute to an adequate nutritional quality and safety analysis of the induced mutants. The consistent metabolic changes observed in the *lpa* soybean mutants compared to the wild-types have to be considered when assessing the nutritional value of the two mutants. Especially the decreased contents of oligosaccharides in *Gm-lpa*-TW75-1 would be a highly desirable trait as they may cause flatulence when consumed by humans [208]. Oligosaccharides cannot be hydrolyzed in the intestine of humans due to the absence of the enzyme α-

galactosidase. For this reason, galactosyl cyclitols are also being discussed to produce flatulence [204].

Except for tyrosine, levels of valine, leucine, serine, ß-alanine, asparagines and citrulline were significantly increased in *Gm-lpa*-TW75-1 compared to the corresponding wild-type. However, at this point it cannot be finally concluded whether these observations result from the intended mutation, from pleiotropic effects or from further mutations independent from the primary mutation event. A single environmental impact seems unlikely as the observed metabolic changes were consistently present at three different field trials within two seasons. A targeted analysis of amino acids in *Gm-lpa*-TW75-1 and the wild-type grown in the spring season 2004 at Hangzhou revealed no statistically significantly amino acid concentrations [209]. However, amino acid concentrations were also slightly increased. Therefore, further studies on the amino acid contents in the wild-type and *lpa* mutant would be necessary.

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

5 SUMMARY

Metabolite profiling represents an approach for the simultaneous detection, identification and quantification of a broad spectrum of polar and non-polar constituents in complex biological systems. The suitability of such an untargeted analysis for the investigation of low molecular weight compounds in induced mutants and in developmental crop systems has been demonstrated in the present study.

A gas chromatography-mass spectrometry (GC-MS) metabolite profiling approach was used to investigate time-dependent metabolic changes in the course of germination and malting of rice and barley, respectively. The crop kernels were incubated for a defined time under ambient conditions. Samples taken during the germination process were subjected to an extraction and fractionation procedure covering fatty acid methyl esters, hydrocarbons, free fatty acids, fatty alcohols, sterols, sugars, sugar alcohols, organic and inorganic acids, amino acids and amines. Investigation of the obtained metabolite profiles by GC resulted in the detection of 615 distinct peaks in rice and of 587 in barley. 174 (rice) and 173 (barley) of them could be identified. Statistical assessment of the data via principal component analysis demonstrated that the metabolic changes during the germination and malting process are reflected by considerable time-dependent shifts of the scores. In addition, relative quantifications based on standardized peak heights revealed dynamic changes of the metabolites in rice and barley in the course of germination.

Three low phytic acid (*lpa*) rice and two *lpa* soybean mutant lines, generated through γ-irradiation, were subjected together with their parent wild-type varieties to a comparative metabolite profiling procedure. High performance liquid chromatography analysis of inositol phosphates in the seeds produced revealed decreased phytic acid ($InsP_6$) contents ranging from -11 to -58 % for rice and from -22 to -66 % for the soybean mutants. Lower phosphorylated inositol phosphates ($InsP_3$ – $InsP_5$) were detected for the *lpa* soybean mutants and the corresponding wild-types. GC metabolite profiling showed that on average 30 % of the detected peaks were statistically significantly different between the wild-types and the *lpa* mutants grown at one field trial. However, only a few of these differences could be consistently observed at all conducted field trials. Identification and quantification of the consistently different metabolites revealed distinct increases and decreases of

compounds which were shown to be related to the biosynthetic pathways leading to phytic acid. Consideration of these metabolic changes in the light of the different routes involved in the biosynthesis of phytic acid allowed a characterization of the different mutants according to their types of mutation underlying a specific phenotype. Moreover, the applied metabolite profiling allowed to distinguish between natural variability and changes in metabolites induced by the mutation event. In addition to metabolite profiling, the influence of mutational breeding on the contents of nutritionally relevant minerals in the *lpa* mutants of rice and soybean was investigated. The analysis of these minerals showed that *lpa* mutations do not result in systematic increases or decreases of mineral contents in such crops.

6 ZUSAMMENFASSUNG

„Metabolite Profiling“ stellt eine Methode dar, welche erlaubt, ein breites Spektrum niedermolekularer Verbindungen in komplexen biologischen Systemen zu detektieren, identifizieren und zu quantifizieren. Die Anwendbarkeit dieser nicht zielgerichteten Analytik sollte im Rahmen der vorliegenden Arbeit anhand der Untersuchungen von Reis- und Sojamutanten mit erniedrigten Gehalten an Phytinsäure sowie von Reis und Gerste, die einer Keimung bzw. Mälzung unterworfen wurden, gezeigt werden.

Für die Untersuchungen von zeitabhängigen metabolischen Veränderungen im Verlauf der Keimung von Reis sowie des Mälzens von Gerste wurde ein auf Gaschromatographie-Massenspektrometrie (GC-MS) basierender Metabolite Profiling Ansatz verwendet. Die Getreidekörner wurden unter definierten Bedingungen inkubiert und nach bestimmten Zeitintervallen einem Extraktions- und Fraktionierungsverfahren unterworfen. Das verwendete Profiling erlaubte die Erfassung eines breiten Spektrums an unpolaren (z.B. Fettsäuremethylester, freie Fettsäuren, Fettalkohole, Sterole, Kohlenwasserstoffe) und polaren (z.B. Zucker, Zuckeralkohole, organische und anorganische Säuren, Aminosäuren, Amine) Inhaltsstoffen. Die GC-Analytik ermöglichte die Detektion von 615 bzw. 587 Peaks in Reis und Gerste. Davon konnten 174 bzw. 173 identifiziert werden. Durch uni- und multivariate Auswertungen der GC Daten konnten die dynamischen Metaboliten-spezifischen Veränderungen im Verlauf der Keimung bzw. des Mälzens erfasst werden.

Die Metabolitenspektren von drei Reis- und zwei Sojamutanten mit erniedrigten Phytinsäuregehalten, generiert durch γ-Bestrahlung, wurden mit denen der zugehörigen Wildtypen mittels Metabolite Profiling verglichen. Eine Untersuchung der Inositolphosphate in den Mutanten mittels Hochleistungs-Flüssigchromatographie ergab reduzierte Phytinsäuregehalte ($InsP_6$) von -11 bis -58 % in Reis und von -22 bis -66 % in Soja im Vergleich zu den entsprechenden Wildtypen. Niedriger phosphorylierte Inositolphosphate ($InsP_3$ – $InsP_5$) wurden nur in den Sojaproben detektiert. Das GC Metabolite Profiling ergab, dass im Durchschnitt 30 % der insgesamt erfassten Peaks in den Reis- und Sojamutanten und deren Wildtypen pro Feldversuch statistisch signifikant unterschiedlich waren. Unter der Berücksichtigung aller Feldversuche konnte jedoch gezeigt werden, dass nur wenige dieser

Unterschiede konsistent waren. Eine Identifizierung und Quantifizierung dieser konsistenten Unterschiede ergab signifikante Gehaltsänderungen von Verbindungen, die in die Biosynthesewege der Phytinsäure involviert sind bzw. von diesen beeinflusst werden. Im Lichte dieser metabolischen Veränderungen konnten die Reis- und Sojamutanten hinsichtlich ihres Mutationstyps charakterisiert und bewertet werden. Weiterhin ermöglichte das Metabolite Profiling eine Differenzierung der Unterschiede, die auf natürliche Variabilität zurückzuführen sind, von denen, die durch die Mutationszüchtung verursacht wurden. Neben der Analytik von niedermolekularen Inhaltsstoffen in diesen Mutanten mittels Metabolite Profiling wurde der Einfluss der Mutationszüchtung auf die Gehalte an ernährungs-physiologisch relevanten Mineralstoffen untersucht. Ein Vergleich der Gehalte in den Mutanten und Wildtypen ergab, dass die aufgrund der Mutation reduzierten Phytinsäuregehalte nicht in systematisch erhöhten oder erniedrigten Mineralstoffgehalten resultierten.

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Publications

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Presentations at meetings of the EU scientific project “Quantitative Risk Assessment Strategies for Novel Foods” (NOFORISK, Projekt Nr. FP6 – 506387, www.noforisk.org).