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# Metabolic changes in transgenic maize mature seeds over-expressing the *Aspergillus niger phyA2*

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## Abstract

**Key message** Non-targeted metabolomics analysis revealed only intended metabolic changes in transgenic maize over-expressing the *Aspergillus niger phyA2*.

**Abstract** Genetically modified (GM) crops account for a large proportion of modern agriculture worldwide, raising increasingly the public concerns of safety. Generally, according to substantial equivalence principle, if a GM crop is demonstrated to be equivalently safe to its conventional species, it is supposed to be safe. In this study, taking the advantage of an established non-target metabolomic profiling platform based on the combination of UPLC-MS/MS with

GC-MS, we compared the mature seed metabolic changes in transgenic maize over-expressing the *Aspergillus niger phyA2* with its non-transgenic counterpart and other 14 conventional maize lines. In total, levels of nine out of identified 210 metabolites were significantly changed in transgenic maize as compared with its non-transgenic counterpart, and the number of significantly altered metabolites was reduced to only four when the natural variations were taken into consideration. Notably, those four metabolites were all associated with targeted engineering pathway. Our results indicated that although both intended and non-intended metabolic changes occurred in the mature seeds of this GM maize event, only intended metabolic pathway was found to be out of the range of the natural metabolic variation in the metabolome of the transgenic maize. Therefore, only when natural metabolic variation was taken into account, could non-targeted metabolomics provide reliable objective compositional substantial equivalence analysis on GM crops.

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**Keywords** GC-MS · Phytase · Safety assessment · Transgenic · Substantial equivalence · UPLC-MS/MS

## Introduction

With the rapid development of modern agricultural biotechnology, more and more genetic modified (GM) crops have been planted by farmers due to their excellent traits such as decreased pesticide use, increased yield, and enhanced herbicide tolerance. So far, 28 countries have planted GM crops, and the planting area of GM crops worldwide increased from 1.7 million hectares in 1996 to 181 million hectares in 2014 (James 2014). In total, 357 GM events covering 27 GM crops have been authorized for food and feed use in 65 countries (James 2014). On the

other hand, the acceptability of GM crops is rather low in some countries owing partly to public concerns about their food safety, environmental risk, and ethical issues, etc. (Dlugosch and Whitton 2008; Zhang and Guo 2011; McClain et al. 2015). As a result, an increasing number of countries and regions have passed strict laws and regulations, requiring detailed risk assessment of GM crops before they are granted for the safety certification and approved for commercial production (FAO/WHO 2000).

A major principle and guiding tool for the risk assessment of a GM crop was the concept of “substantial equivalence”, which was introduced by Organization for Economic Cooperation and Development (OECD) in the early 1990s (Schauzu 2000; Kuiper et al. 2001). The guiding tool was supposed to determine whether the GM food is as safe as its traditional species, focusing on critical factors including nutritional composition, potential toxicity, potential allergenicity and possible secondary effects (Schauzu 2000; Kuiper et al. 2001; Ladics et al. 2014). As a result, comparative compositional studies on both key nutrients (such as carbohydrates, fatty acids, amino acids, minerals, and vitamins) and anti-nutrients (phytic acid, protease inhibitor and lipoxidase) have been considered extensively in substantial equivalence studies of GM crops. Up to now, various GM crops, such as soybean, corn, wheat, potato, tomato, pea, cotton, and rice, have been subjected to these analyses (Ricroch et al. 2011; Gayen et al. 2013; Iwaki et al. 2013; Wang et al. 2015). For example, comparative analysis of nutritional compositions was performed between transgenic high iron rice with its non-transgenic counterpart (Gayen et al. 2013). The results suggested no significant differences of nutritional and anti-nutritional components between the transgenic ferritin rice and the non-transgenic IR68144 rice except certain intended changes due to the inserted ferritin gene. The targeted approaches of substantial equivalence studies for GM crops have limitations regarding identification of unknown anti-nutrients and natural toxins, and the process was more or less complex, low throughput and time consuming. Therefore, the so called “-omics” technologies including transcriptomics, proteomics, and metabolomics, as non-targeted approaches have recently been used for substantial equivalence studies of GM crops (Rocco et al. 2008; García-Cañas et al. 2011; Montero et al. 2011). For example, using microarray hybridization, around 0.40 % transcriptomic differences were found in leaves of transgenic rice lines expressing an antifungal protein (Montero et al. 2011). As for proteomic profiling, Rocco et al. found that a number of proteins differently changed in a GM tobacco transformed with the tomato prosystemin gene, which is involved in oxidative stress, protection from pathogens and in carbon/energy metabolism (Rocco et al. 2008). Moreover, gas chromatography–mass spectrometry (GC–MS) analysis of metabolites profiling of potato tubers that were genetically

modified to contain more efficient sucrose catabolism revealed a significant elevation in the content of each individual amino acid (García-Cañas et al. 2011). The “-omics” technologies are unbiased larger-scale analyses and stringent in the assessment of the potential for any unintended effects. Among the “-omics” approach, transcriptomics and, to a lesser extent, proteomics were used while metabolomics was the prevalently used one since the metabolites (both primary and secondary metabolites) are more relevant to the plant phenotype and nutritional and toxicological characteristics (Harrigan and Chassy 2012). Besides, toxic and allergenic properties are also considered as focal aspects in substantial equivalence studies for GM crops and the comprehensive assessment for both toxicants and allergens was included in the Codex food safety guideline (Alimentarius 2003; McClain et al. 2015).

The transgenic maize over-expressing *Aspergillus niger phyA2* used in this study was developed in China, which produces significant quantities of the active phytase in the seed; consequently, it reduces the phytic acid content (around 23 %) and increases the inorganic phosphate (Pi) content (about 3-fold) in transgenic seeds (Chen et al. 2008). Therefore, it eliminates the need for phosphorus supplementation of monogastric animal feed and reduces the adverse impact of animal pollution on the environment. Although this transgenic maize is of high potential for commercial production, the concern about its food safety derived from the uncertain of the new technology has arisen in a parallel manner. In our previous transcriptomic study, digital gene expression (DGE) analysis revealed the co-occurrence of both intended and unintended transcriptomic changes in this transgenic event, which highlighted the need for further investigations into the event to completely fulfill the risk assessment (Rao et al. 2013).

In this study, taking the advantage of an established powerful and global unbiased metabolomic profiling platform, we profiled the mature seed metabolome of the transgenic maize over-expressing the *Aspergillus niger phyA2* and its non-transgenic counterpart, along with 13 elite inbred and one hybrid maize lines (Supplemental Table S1), with the aim to provide detailed metabolomic characteristics of this transgenic event for its further risk assessment.

## Materials and methods

### Materials

The seeds of transgenic maize over-expressing the *Aspergillus niger phyA2* gene and its non-transgenic counterpart were kindly provided by Dr. Rumei Chen from Biotechnology Research Institute, Chinese Academy of Agricultural Sciences. Other fourteen conventional maize lines were

kindly provided by Prof. Jinsheng Lai from China Agricultural University. All maize lines were planted at a farm in Minhang, Shanghai, China. The experimental design was a randomized complete block design including three repeats for each line. All primary ears were self-pollinated. All primary ears from each plot were harvested at physiological maturity, and air dried under sunlight for 72 h, frozen with liquid nitrogen and kept at  $-80\text{ }^{\circ}\text{C}$  until analysis.

### Metabolic profiling

Metabolic profiling of maize seeds was performed using a global unbiased platform, a combination of three independent analytical platforms: UPLC/MS/MS optimized for basic species, UPLC/MS/MS optimized for acidic species, and GC/MS. The detailed information for these platforms and the process of metabolites profiling including sample extraction was reported in our previous study (Rao et al. 2014).

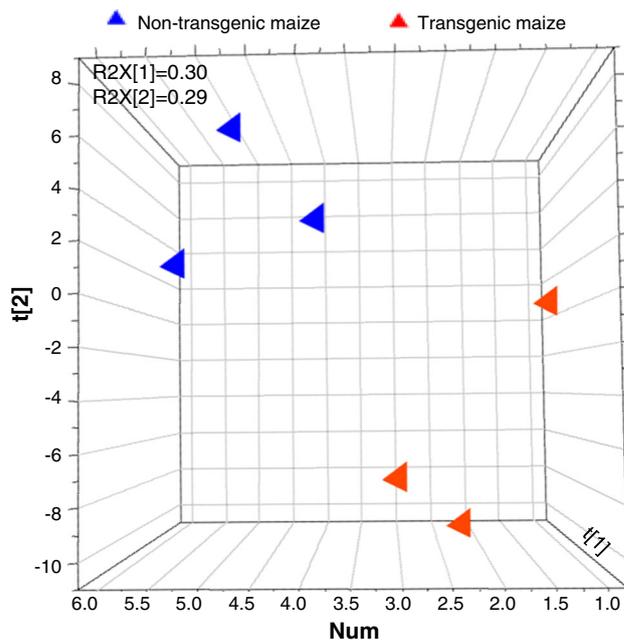
### Data analysis

Integrated peak ion counts were used to compare relative abundances of a metabolite in each sample. The missing values for a given metabolite were imputed with the observed minimum detected value for statistical analysis, assuming that they were below the limits of instrument detection sensitivity. Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were done with SIMCA-P 12.0 software and significant changed metabolites were determined with SPSS 17.0 software (Rao et al. 2014). Before Z score analysis, metabolites whose contents are the same values in more than half of the tested lines (7 lines in this study) were filtered for their highly suspect undetectable as mentioned above. The remained 193 metabolites were log transformed and the Z scores of 16 maize lines (including 14 conventional maize lines, transgenic and non-transgenic maize lines) were calculated as follows:  $(X - \text{Mean of the 14 conventional maize lines}) / \text{Standard deviation of 14 conventional maize lines}$ . Here, X was the transformed data for each metabolite. The natural variation of each metabolite in maize seed was represented by Z plot with 3 SD as cutoff constructed with R. Usually, elemental compositions with a Z value greater than 3 were labeled as outliers (Gupta et al. 2003; Garcia 2012).

## Results

### Metabolic profiling of transgenic and non-transgenic maize mature seed

To perform metabolite profiling, the global unbiased metabolic profiling platform (a combination of GC/MS and



**Fig. 1** PCA model of the metabolic data of transgenic and non-transgenic mature maize seeds. Samples of transgenic maize were clearly separated from those of non-transgenic maize samples

LC/MS/MS) was used (Evans et al. 2009; Ohta et al. 2009). In total, the same 210 metabolites were identified in both transgenic and non-transgenic mature maize seeds, which covered all the eight major pathways and contained 64 amino acids, 55 carbohydrates, 37 lipids, 18 compounds in the cofactor/electron carrier class, 21 nucleotide derivatives, 4 peptides, 2 phytohormones and 9 secondary metabolites, according to the database from Plant Metabolic Net (PMN) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Supplemental Table S2). It uncovered so far the most broad maize seed metabolome as compared with previous studies (Harrigan et al. 2007; Skogerson et al. 2010; Frank et al. 2012; Yang et al. 2013), and provided abundant data for the determination of differences between transgenic maize and its non-transgenic line.

The widely used unsupervised method, principal component analysis (PCA), was firstly performed for transgenic and non-transgenic maize for all 210 metabolites. As shown in Fig. 1, transgenic maize seeds could be completely separated from non-transgenic lines. And then the supervised method PLS-DA was employed to identify metabolites responsible for the separation of transgenic and non-transgenic lines. Together with independent t tests (SPSS 17.0), the statistical analysis showed that 9 metabolites (Table 1; Fig. 2a, b) were significantly changed ( $p \leq 0.05$ ), playing an important role in the separation. These metabolites contained 8 increased and 1 decreased compounds involved in 6 super pathways and 9 sub pathways. The only decreased metabolite was *myo*-inositol

**Table 1** List of different metabolites between transgenic and non-transgenic lines, responsible for the separation

Super pathway	Sub pathway	Biochemical	Fold change (transgenic/non-transgenic)	P value
Amino acid	Aromatic amino acid metabolism (PEP derived)	Tyrosine	3.15	0.032
Carbohydrate	Amino sugar and nucleotide sugar	Glucosamine	2.31	0.012
	Inositol metabolism	IP6	0.02	0.049
	Sucrose, glucose, fructose metabolism	Raffinose	1.69	0.037
	TCA cycle	Citrate	3.25	0.045
Cofactors, Prosthetic Groups, Electron Carriers	Oxidative phosphorylation	Phosphate	2.70	0.046
Hormone metabolism	Auxin metabolism	Indole-3-acetyl-aspartate	2.00	0.021
Lipids	Free fatty acid	2-hydroxyglutarate	2.03	0.023
Peptide	Gamma-glutamyl	Gamma-glutamyltyrosine	2.99	0.007

hexakisphosphate (IP6) that was reduced by 98 %, while phosphate was reversely increased to 2.70 times in transgenic maize when compared with non-transgenic maize. The detected IP6 was much lower although the increased phosphate was consistent with the previous report (Chen et al. 2008). Other 7 significantly increased metabolites ranged from 1.69 to 3.25 folds.

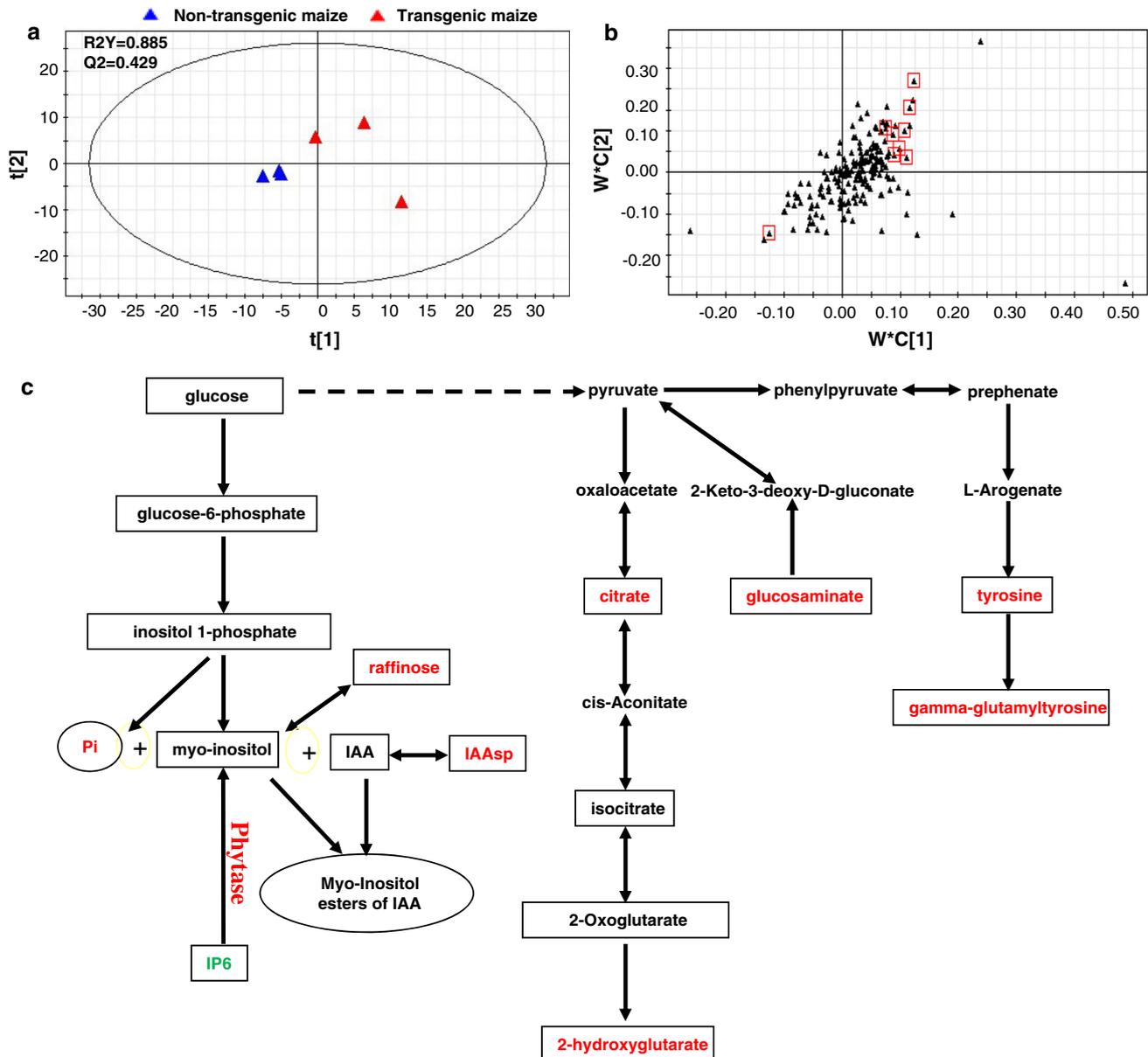
#### Effects of overexpressing the *Aspergillus niger phyA2* on the maize seed metabolic pathways

To fully understand the changed pathways in transgenic mature maize seed, a simple network was constructed based on differentially changed metabolites, which revealed that at least six of these are directly or indirectly linked to the targeted pathway (Fig. 2c): the hydrolysis of IP6. Previous reports demonstrated the specific expression of *phyA2* gene in the transgenic maize by qualitative PCR (Rao et al. 2013). Accordingly, IP6 was metabolized into Pi as expected with the enzyme, which led to the decrease of the content of IP6 and the increase of the content of Pi. The changed content of Pi in transgenic maize probably led to the altered content of tyrosine and then influenced gamma-glutamyltyrosine level (Berger et al. 1996; Kameyama et al. 2000). In *myo*-inositol (MI) biosynthesis, conversion of glucose to *myo*-inositol, the referred four metabolites, glucose, glucose-6-phosphate, inositol 1-phosphate, *myo*-inositol, all remained to be constant. Notably, the levels of two metabolites, raffinose and indole-3-acetyl-aspartate (IAAsp), both went up likely due to *myo*-inositol, though that of *myo*-inositol remained to be constant. Previous studies have already confirmed that both raffinose and IAAsp are tightly associated with *myo*-inositol. For example, the reduction in the *myo*-inositol level reduced

galactinol and raffinose family oligosaccharides (RFO) levels drastically in mutant soybean seeds that confers a decreased raffinose and phytic acid phenotype (Hitz et al. 2002). There was highly significant correlation between the levels of *myo*-inositol and RFOs in pea seeds (Karner et al. 2004). Previous studies also showed that about one-half of the IAA was present as high molecular weight esters with *myo*-inositol in kernels of maize and could also conjugate into IAAsp (Ueda and Bandurski 1969; Sitbon et al. 1993). The other unintended changes of the left 3 metabolites (glucosamine, citrate, 2-hydroxyglutarate) seemed to be associated with stress response (Gechev and Hille 2012). Thus, both intended and non-intended metabolic changes were observed in the transgenic maize line.

#### Comparison of GM-induced metabolic changes in transgenic mature seeds with natural variation in non-transgenic conventional mature seeds

In the maize lines examined in this study, PCA of all tested maize lines showed that the metabolite profiles of both transgenic and non-transgenic maize lines fell within the range of conventional lines examined (Fig. 3a). On the other hand, Z scores, a very popular and ideal tool for extracting statistically significant information from widely scattered data points that have been used to label outliers in report elemental compositions in each spice category as well as different flavors and packages (Gupta et al. 2003; Garcia 2012), showed that 5 metabolites in transgenic maize seed were observed to be outliers from conventional lines; they were IP6, inositol 2-phosphate (I2P), methylphosphate, 5-methylthioadenosine (MTA), and inositol 1-phosphate (I1P) (Fig. 3b). While in non-

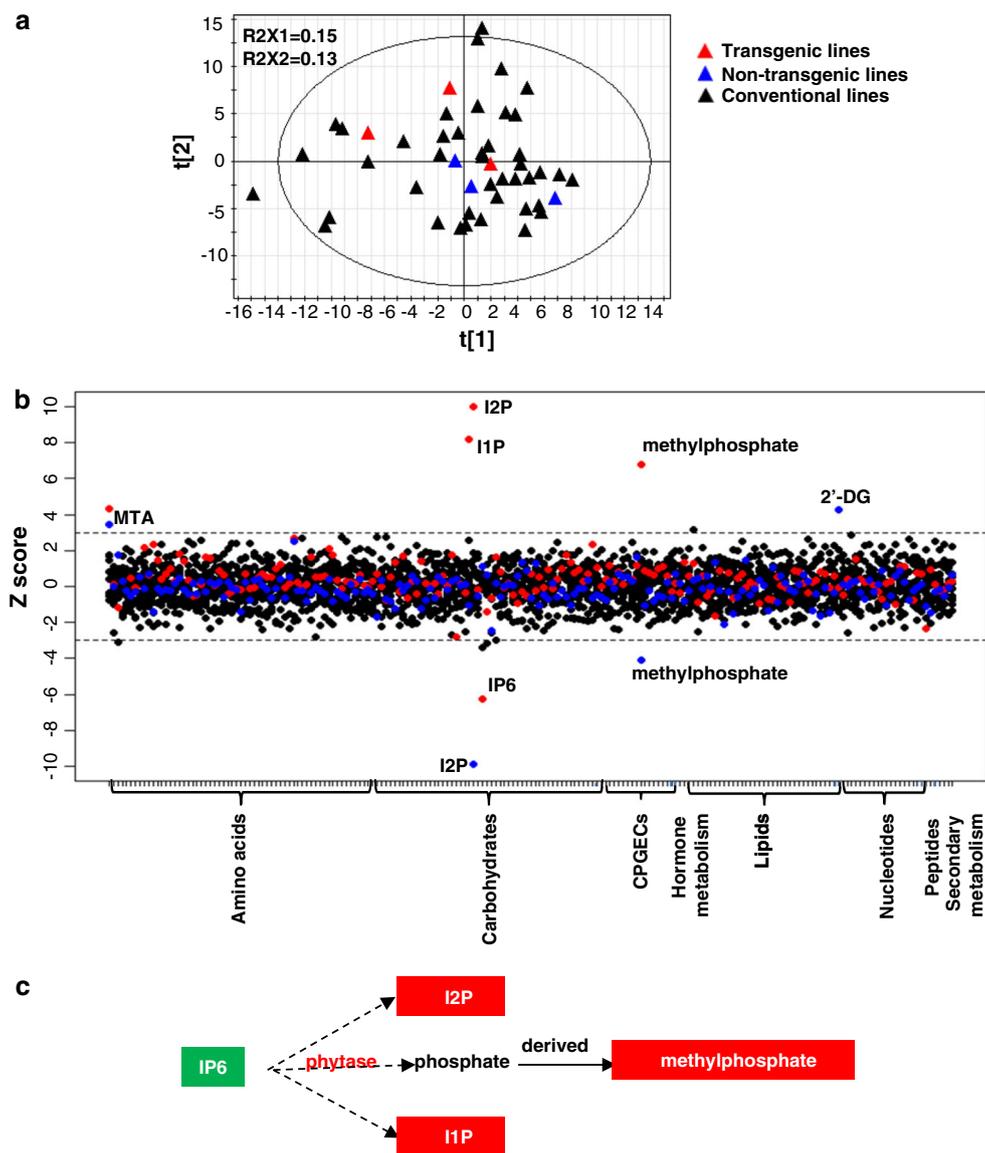


**Fig. 2** Differential metabolites and involved networks of transgenic maize over-expressing the *Aspergillus niger phyA2* gene and non-transgenic maize. **a** Score plot from PLS-DA model of transgenic and non-transgenic maize. **b** Loading plot from PLS-DA model of transgenic and non-transgenic maize. Metabolites playing key roles for separation are marked with red squares. **c** Pathway constructed by changed metabolites indicating intended and unintended effects in

transgenic maize seeds. The detected metabolites were represented in box while metabolites labeled in red or green represented increased or decreased, respectively, in transgenic maize seeds. *Pi* inorganic phosphate; *IAA* indoleacetate; *IAAsp* indole-3-acetyl-aspartate, *IP6* myo-inositol hexakisphosphate, *IP5* myo-inositol pentakisphosphate (1,2,4,5,6 or 1,3,4,5,6); *IP4*, myo-inositol tetrakisphosphate (1,3,4,6 or 3,4,5,6 or 1,3,4,5); *IP3*, myo-inositol triphosphate (1,4,5 or 1,3,4)

transgenic counterpart maize, 4 metabolites were outliers; they were I2P, methylphosphate, MTA, and 2'-deoxyguanosine (2'-DG). As a result, only four metabolites in transgenic maize were especially outliers; they were IP6, I2P, I1P and methylphosphate. The overexpression of *phyA2* reduced the level of IP6 significantly as expected in transgenic maize, and led to the increase of Pi, I1P and I2P, and methylphosphate is likely derived from

methylation of increased phosphate dehydrolized from IP6 during GC–MS derivation (Fig. 3c). Therefore, all these four outliers were associated with targeted engineering pathway. The result here indicated only intended metabolic changes in this transgenic maize event when taken natural variation into consideration, which was similar with the recent study on transgenic soybean (Clarke et al. 2013).



**Fig. 3** **a** PCA model of transgenic, non-transgenic and 14 conventional maize lines. **b** Z score plot of metabolites in all maize lines including transgenic maize and non-transgenic maize. **c** The targeted engineering pathway for transgenic event: a lower level of IP6, and

higher levels of I2P, I1P and methylphosphate. Metabolites in red or green box increased or decreased, respectively, in transgenic maize seeds. IP6 myo-inositol hexakisphosphate, I2P inositol 2-phosphate, I1P inositol 1-phosphate, 2'-DG 2'-deoxyguanosine

### Discussion

Up to now, various types of genetically engineered traits have been introduced into GM plants including four major crops: soybean, maize, rapeseed, and cotton. Herbicide (glyphosate, glufosinate and oxynil) tolerance and insect resistance (various forms of *Bacillus thuringiensis* Cry proteins) traits dominated the current planted GM crops. Newer introduced traits such as ripening delay, provitamin A enrichment, and drought tolerance have been developed to produce valuable compounds or increase yield in an

economically attractive format by altering metabolic output. For example, Stark et al. (1992) overexpressed a deregulated bacterial AGPase to increase the activity of the starch biosynthetic pathway in potato tubers and the transformed lines have an average of 35 % more tuber starch than the controls (Stark et al. 1992). In this study, the transgenic maize event was engineered to produce exogenous phytase to catalyze the hydrolysis of IP6 and release Pi. As expected, non-targeted metabolites profiling revealed that those four outlier metabolites resulted directly from the target-engineered pathway in transgenic maize

seed when natural variation was taken into consideration. Our results, for the first time, revealed the molecular characterization of this transgenic maize at the metabolomic level.

Previous safety assessment studies on GM crops mainly employed one-to-one comparative compositional analysis based on targeted or non-targeted metabolomics approaches (Baker et al. 2006; Barros et al. 2010; Coll et al. 2010; Harrigan et al. 2010; Ricoch et al. 2011), as the composition of compounds detected in metabolome was closely related to the phenotype of the organism, and especially associated with important nutritional and toxicological characteristics (Kusano et al. 2011). Because most of the analyses were performed on only one metabolomic platform, either GC–MS, or liquid chromatography–mass spectrometry (LC–MS), or nuclear magnetic resonance (NMR), and because the lack of standard references in plants, limited metabolites were detected and identified, which hindered the effective and comprehensive compositional safety assessment of GM crops. In this study, each biological replicate (one sample) was simultaneously analyzed three times by GC–MS, UPLC–MS/MS (positive) and UPLC–MS/MS (negative), and the resulting signals were search against a commercial database of about 1500 standard metabolites; 210 metabolites in total were identified in each maize sample, which uncovered so far the most broad maize seed metabolome as compared with previous studies (Harrigan et al. 2007; Skogerson et al. 2010; Frank et al. 2012; Yang et al. 2013). It is worthy to note that nine secondary metabolites were also identified in the study, two of them, dihydrokaempferol and naringenin, are flavonoids associated with biological processes in plants such as pigmentation of flowers, fruits and vegetables, fertility, plant–pathogen interactions, and protection against UV light (Bovy et al. 2010). Recent studies found that flavonoids protect humans against oxidative stress, certain cancers, coronary heart disease, and other age-related diseases (Rahman 2007). Currently, very limited secondary metabolites were listed with compositional considerations for new varieties of maize including transgenic events, with the exception of furfural, ferulic acid and *p*-coumaric acid (OECD 2002; Herman et al. 2007). Though furfural was not detected in our study, other nine secondary metabolites including ferulic acid and *p*-coumaric acid were included. Hence, it represented so far the most comprehensive and broad metabolic study for GM maize. These metabolites could be used to develop a maize seed metabolome database for future evaluation of GM maize seeds with different traits. It is believed that the more secondary metabolites characterized in plants, the more potential for the identification of unintended effects by genetic modification in GM crops.

Various approaches can be used to compare the changes in transgenic events with natural variations in the comparative compositional assessment of GM crops. A simple method is to determine whether each compound level is within the safe ranges for food defined by international/national organizations (such as OECD, FAO) (Harrigan et al. 2010). However, the absolute quantitative methods for large amount of metabolites in crops are limited and time consuming; it requires a lot of samples and needs continuous complementation of novel metabolites into the database; therefore, it is not suitable for high-throughput identification for safety assessment studies of GM crops. Other approaches included PCA, Kohonen self-organizing map (SOM), and analyses of variance (ANOVA) were also included, but no agreement was achieved for the evaluation of following-up transgenic events (Shepherd et al. 2006; Coll et al. 2010). In this study, PCA combining with *Z* scores could simultaneously determine whether each transgenic sample was within the conventional samples and whether each metabolite was within the ranges of nature variations. Accordingly, with the metabolite database based on this study, the metabolic study of novel transgenic maize seed could be performed effectively using the same strategy.

In conclusion, the combination of UPLC–MS/MS with GC–MS could provide a broader maize metabolome analysis, which could be employed to comparatively study the substantial equivalence of the transgenic maize over-expressing the *Aspergillus niger phyA2* and its non-transgenic counterpart in relation to natural metabolic variation. This study also provides metabolites database and evaluation strategy for safety assessment studies of other transgenic event of maize seed.

**Author contribution statement** Rao J carried out most of the experiments and data analysis, and wrote the first draft of the manuscript. Yang L, Guo J, and Quan S helped in the data analysis, Zhao X, and Chen G assisted in performing experiments. Shi J and Zhang D designed the experiment and revised the manuscript.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

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