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# Metabolic profiling of maize seeds with both insect- and herbicide-resistance genes (*cry1Ab* and *epsps*), dual herbicide-resistance genes (*epsps* and *pat*), and natural genotypic varieties

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## Abstract

**Background:** Widely targeted metabolomics was applied to estimate the differences in the metabolite profiles of maize seeds from 3 natural genotypic varieties and 4 genetically modified (GM) lines.

**Results:** Pairwise comparison with their isogenic controls revealed 71, 121, 43 and 95 differentially accumulated metabolites (DAMs) in GM maize seeds of C0030.2.4, C0030.3.5, C0010.1.1 and C0010.3.1, respectively. KEGG pathway enrichment analysis showed that most of these DAMs participated in the biosynthesis of secondary metabolites and purine metabolism in GM maize C0030.2.4 and C0030.3.5, but participated in tryptophan metabolism and 2-oxocarboxylic acid metabolism in C0010.3.1 seeds and in metabolic pathways and the biosynthesis of secondary metabolites in C0010.1.1 seeds. The data also showed that the differences in metabolite accumulation, both total DAMs and co-DAMs, among the different natural genotypic varieties (418 DAMs and 39 co-DAMs) were greater than those caused by genetic modification (330 DAMs and 3 co-DAMs).

**Conclusions:** None of the DAMs were identified as new or unintended, showing only changes in abundance in the studied maize seeds. The metabolite profile differences among the 3 non-GM lines were more notable than those among GM lines. Different genetic backgrounds affect metabolite profiling more than gene modification itself.

**Keywords:** Widely targeted metabolomics, Differentially accumulated metabolites, Unintended effects, Genetic modification, Natural genotypic varieties, Maize seeds

## Introduction

The commercialization of genetically modified (GM) crops has not only led to considerable economic gains but also reduced environmental problems. However, due to unintended effects, GM crops remain a focus of debate [1, 2]. Therefore, the overall safety of GM crops must be evaluated [3–6]. Omics (e.g., genomics, transcriptomics,

proteomics, and metabolomics) methods developed in recent years enable unbiased safety assessments and provide comprehensive and detailed insights into unintended changes in studied GM crops [7, 8]. Compared to genomics and transcriptomics, proteomics and metabolomics can be used to better evaluate the levels of nutrients and other food quality and safety factors [9]. Therefore, isobaric tags with relative and absolute quantitation (iTRAQ)-based proteomics have been widely used in the safety evaluation of GM crops [10–15]. Our previous study also showed that this method can successfully

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assess unintended changes in GM maize [16]. In addition to proteins, metabolites not only influence crop growth, development and reproduction [17, 18], but also largely affect crop quality traits [19]. Recently, advances in metabolite profiling technology have made it possible to comprehensively compare metabolite differences in crops affected by growth environments [9], genetic engineering and conventional cross-breeding [20–24]. A recent study has even shown that 10 times more DAMs were observed under different growing environments [25].

Insect resistance and herbicide resistance are the main features of GM crops. The *Bacillus thuringiensis* (*Bt*) *cry* gene (encoding the Cry protein exhibiting high selectivity and a broad insecticidal spectrum) has been widely used in GM crops [26]. Among herbicide-resistance genes, the *epsps* (encoding 5-enolpyruvate shikimic acid-3-phosphate synthase, EPSPS) [27–29] and *bar/pat* (encoding phosphinothricin acetyl transferase, PAT) [30, 31] genes are most widely used.

Recently, published data have analysed the effects of HIR (herbicide- and insect-resistance) gene cassettes and different growing environments on the transgenic maize metabolome [25]. In this study, widely targeted metabolomics was used for the analysis of maize seeds from 4 GM maize lines, with two carrying both insect- and herbicide-resistance genes (C0030.2.4 and C0030.3.5) and two with dual insertion of herbicide-resistance genes (C0010.3.1 and C0010.1.1), and 3 non-GM maize lines (NH101, DH605 and NH106) to fill the knowledge gaps concerning metabolite differences in GM and natural genotypic varieties and enrich the field of GM and non-GM maize metabolomics.

## Materials and methods

### Plant materials

Seeds of the GM maize lines C0030.2.4 (A1; carrying *epsps* and *cry1Ab* genes), C0010.3.1 (A2; carrying *epsps* and *pat* genes), C0030.3.5 (B1; carrying *epsps* and *cry1Ab* genes) and C0010.1.1 (C2; carrying *epsps* and *pat* genes) and the corresponding non-GM isogenic varieties NH101 (A0), DH605 (B0) and NH106 (C0) were selected for study. The details of these maize lines were described previously [16]. Maize seeds with full grains and uniform sizes were provided by Dabeinong Co. The GM maize lines and their non-GM parents were collected from the same natural growth environment and stored at  $-80^{\circ}\text{C}$ .

### Metabolite preparation

Six biological replicates of seeds of the 7 studied maize lines were used for metabolite profiling. Maize seed grains of each line were ground in liquid  $\text{N}_2$ . The total metabolites were extracted from 100 mg of seed powder with 70% aqueous methanol overnight at  $4^{\circ}\text{C}$ . Following

centrifugation at  $10,000g$  for 10 min, the extracts were absorbed by a CNWBOND Carbon-GCB SPE Cartridge (ANPEL, Shanghai, China) and filtered before UPLC (ultra-performance liquid chromatography)–MS/MS (mass spectrum) analysis.

### UPLC conditions and ESI-QTRAP-MS/MS

The extracted metabolites were analysed using a UPLC–ESI-MS/MS system (UPLC, Shim-pack UFLC Shimadzu CBM30A system; MS, Applied Biosystems 4500 Q TRAP). The analytical conditions and ESI-QTRAP-MS/MS operating parameters were described previously [32].

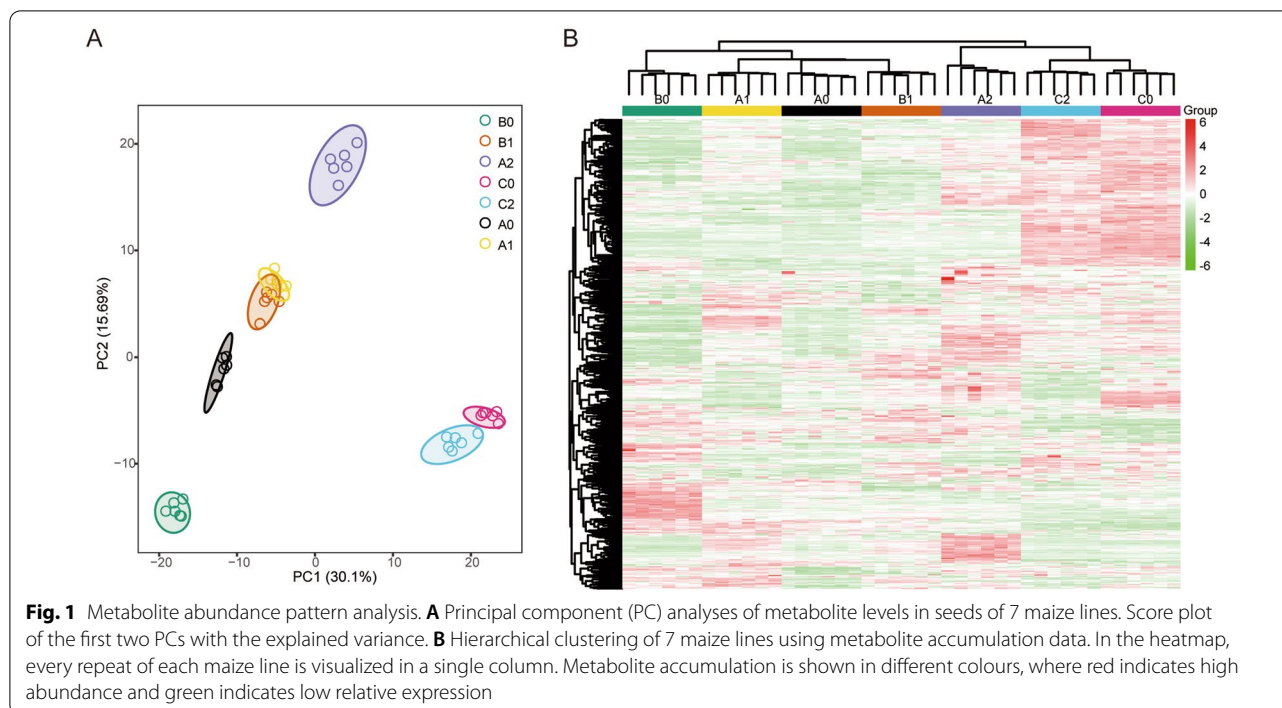
### Metabolite data analysis

Quality control (QC) analysis was conducted to confirm the reliability of the data. A QC sample was prepared by mixing sample extracts and inserted into every two samples to monitor changes over repeated analyses. Orthogonal partial least squares-discriminant analysis (OPLS-DA) was used to maximize the metabolome differences by removing irrelevant differences between the GM and non-GM parent samples. Based on the OPLS-DA results, the metabolites of different maize samples were preliminarily screened using the variable importance in projection (VIP) values of the obtained multivariate OPLS-DA model. The P-value and fold change values of the univariate analysis were combined to further screen DAMs. Metabolites with a  $\text{VIP} \geq 1$  and a fold change  $\geq 2$  or  $\leq 0.5$  were considered differential metabolites for group discrimination [33]. Principal component analysis (PCA) was performed using the statistics function *prcomp* within R ([www.r-project.org](http://www.r-project.org)). A heatmap based on hierarchical cluster analysis was generated in R software ([www.r-project.org](http://www.r-project.org)). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation of the DAMs was carried out using the KEGG database <http://www.genome.jp/kegg/> [34, 35].

## Results

### Metabolite profiling of maize seeds

In total, 664 metabolites were successfully detected in maize seeds by widely targeted metabolomics (Additional file 1: Table S1). The detected metabolites were diverse and could be classified into 31 classes, including amino acids and derivatives, nucleotides and derivatives, phenolic acids, organic acids, sugar alcohols, etc. PCA yielded PC1 (30.1%) and PC2 (15.69%) as the two main components. Repeated samples of the 7 maize lines clustered together well (Fig. 1A). Cluster analysis of the identified metabolites showed that the metabolite profiles of the 7 maize lines were divided into two groups as follows: B0, A1, A0, and B1 and A2, C2, and C0. The metabolite profiles of A0 and B1 shared the highest similarity among



the first group and shared higher similarity with those of A1 and A0 than those of B1 and B0. The metabolite profiles of C2 and C0 shared higher similarity than those of A2 and C0 did (Fig. 1B).

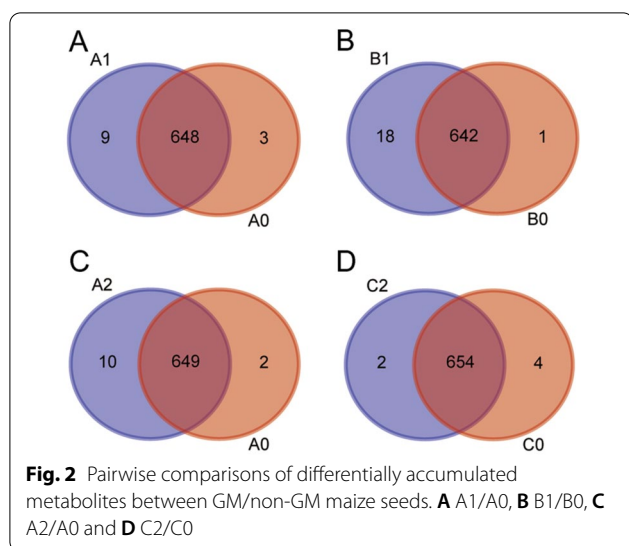
#### Identification of DAMs and co-DAMs in maize seeds

Metabolites with changes in abundance greater than 2.0-fold or lower than 0.5-fold ( $VIP \geq 1$ ) were identified as DAMs. The numbers of DAMs in the different comparison groups are summarized in Table 1. There were 71 DAMs identified in the A1/A0 sample, including 59 upregulated metabolites and 12 downregulated metabolites (Additional file 1: Table S2). One hundred twenty-one DAMs were identified by the comparison of B1 with B0, of which 86 were upregulated and 35 were

downregulated (Additional file 1: Table S3). A total of 95 DAMs were identified in the A2/A0 sample, including 88 upregulated metabolites and 7 downregulated metabolites (Additional file 1: Table S4). There were 43 DAMs identified in the C2/C0 sample, including 12 upregulated metabolites and 31 downregulated metabolites (Additional file 1: Table S5). In total, 330 DAMs were identified in four GM/non-GM comparison groups (Table 1). There were 112, 169 and 137 DAMs identified in the A0/B0, B0/C0 and A0/C0 samples, respectively (Additional file 1: Tables S6–8). In total, 418 DAMs were identified in three non-GM/non-GM comparison groups (Table 1). Pairwise comparisons revealed that there were 9, 18, 10 and 2 new metabolites in A1/A0, B1/B0, A2/A0 and C2/C0, respectively

**Table 1** Summary of the numbers of DAMs in different comparison groups

Comparison groups	No. of upregulated metabolites	No. of downregulated metabolites	No. of DAMs	Total no. of DAMs
A1/A0	59	12	71	330
B1/B0	86	35	121	
C2/C0	12	31	43	
A2/A0	88	7	95	
A0/B0	58	54	112	418
B0/C0	39	130	169	
A0/C0	19	118	137	



(Fig. 2A–D). These new metabolites were new only to their non-GM parents, but were present in other non-GM maize lines (Additional file 1: Figure S1).

#### Analysis of the identified DAMs of A1/A0 and B1/B0 resulting from the insertion of both insect- and herbicide-resistance genes (*cry1Ab* and *epsps*)

KEGG pathway analysis showed that the DAMs of A1/A0 were annotated to 22 KEGG pathways. Biosynthesis of secondary metabolites (ko01110) and purine metabolism (ko00230) were the primary enriched pathways, followed by flavone and flavonol biosynthesis (ko00944) (Fig. 3A). The DAMs of B1/B0 were annotated to 38 KEGG pathways. Biosynthesis of secondary metabolites (ko01110) and purine metabolism (ko00230) were the primary enriched pathways, followed by pentose and glucuronate interconversions (ko00040) (Fig. 3B).

#### Analysis of the identified DAMs of A2/A0 and C2/C0 resulting from the dual insertion of herbicide-resistance genes (*epsps* and *pat*)

KEGG pathway analysis showed that the DAMs of A2/A0 were annotated to 45 KEGG pathways. Tryptophan metabolism (ko00380) and 2-oxocarboxylic acid metabolism (ko01210) were the primary enriched pathways, followed by tyrosine metabolism (ko00350) (Fig. 4A). The DAMs of C2/C0 were annotated to 18 KEGG pathways. Metabolic pathways (ko01100) and biosynthesis of secondary metabolites (ko01110) were the primary enriched pathways, followed by valine, leucine and isoleucine biosynthesis (ko00290) (Fig. 4B).

#### co-DAMs identified in seeds of all four GM and three non-GM maize lines

Among the identified DAMs, Lmhp003013, pme3961 and Lmyp004081 were simultaneously identified in four comparison groups, A1/A0, B1/B0, A2/A0 and C2/C0, and designated co-DAMs (Fig. 5A). Only pme3961 was involved in purine metabolism and metabolic pathways (Table 2). The regulatory trends of these co-DAMs are shown in Fig. 5B. These co-DAMs were downregulated in GM maize line C2 seeds but upregulated in GM maize line A1 and B1 seeds. Lmhp003013 and Lmyp004081 were upregulated in seeds of the GM maize line A2, but pme3961 was downregulated. In the non-GM maize comparison groups A0/B0, B0/C0 and A0/C0, 39 co-DAMs were identified (Fig. 5C). Their regulatory trends are shown in Fig. 5D. pme3961 was also identified as a co-DAM in three non-GM maize comparison groups.

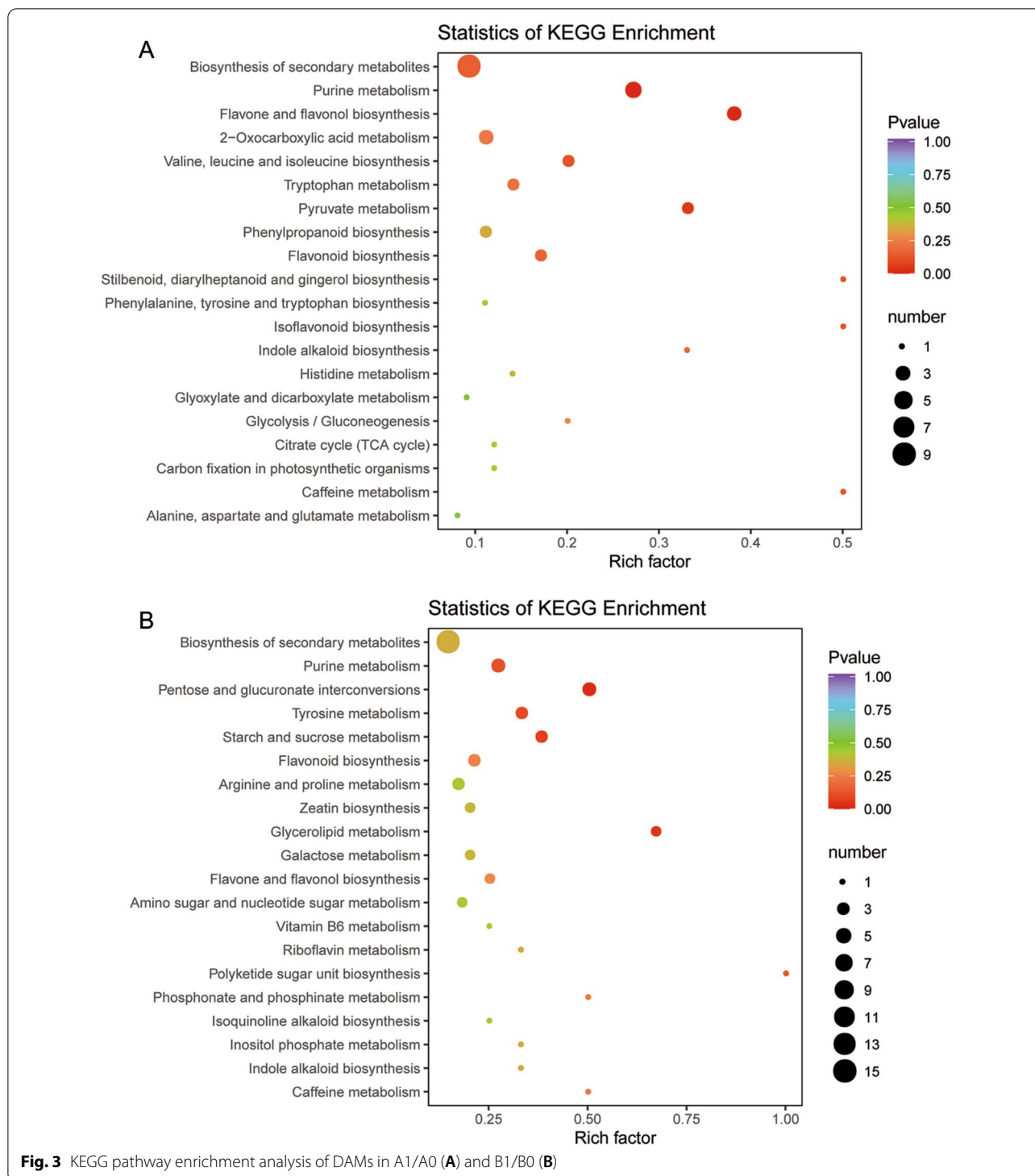
#### Integrated proteomics and metabolomics analyses

Integrated proteomics and metabolomics analyses showed that DEPs [16] were involved in 61, 33, 72 and 72 KEGG pathways in the A1/A0, B1/B0, A2/A0 and C2/C0 comparison groups. DAMs were involved in 24, 38, 45 and 18 KEGG pathways in the A1/A0, B1/B0, A2/A0 and C2/C0 comparison groups. These identified DEPs and DAMs participated in 14, 14, 31 and 13 common KEGG (co-KEGG) pathways in the four comparison groups (Table 3). The analyses also showed that only the DEPs and DAMs in GM maize A2 seeds were simultaneously annotated in phenylalanine, tyrosine and tryptophan biosynthesis (Ko00400). However, the introduction of exogenous EPSPS did not affect the expression levels of six other enzymes, or cause an increase in abundance of seven metabolites (Additional file 1: Figure S2).

#### Discussion

Safety assessments of GM crops are performed to demonstrate that these crops are substantive equivalents to their non-GM isogenic controls to ensure consumer health and safety. Therefore, nontargeted methods of substantive equivalence analysis are the best ways to evaluate the safety of GM crops. Metabolites accumulate in seeds, largely affecting crop quality traits [19]. Here, metabolite profiling of maize seeds was performed by widely targeted metabolomics to facilitate the study of alterations in metabolite accumulation regulated by transgenic modifications and/or different natural genotypic variations. Four GM maize lines were used to study the effects of genetic modification with the same and/or different genetic backgrounds, while three natural genotypic maize varieties were studied to understand the metabolite differences among



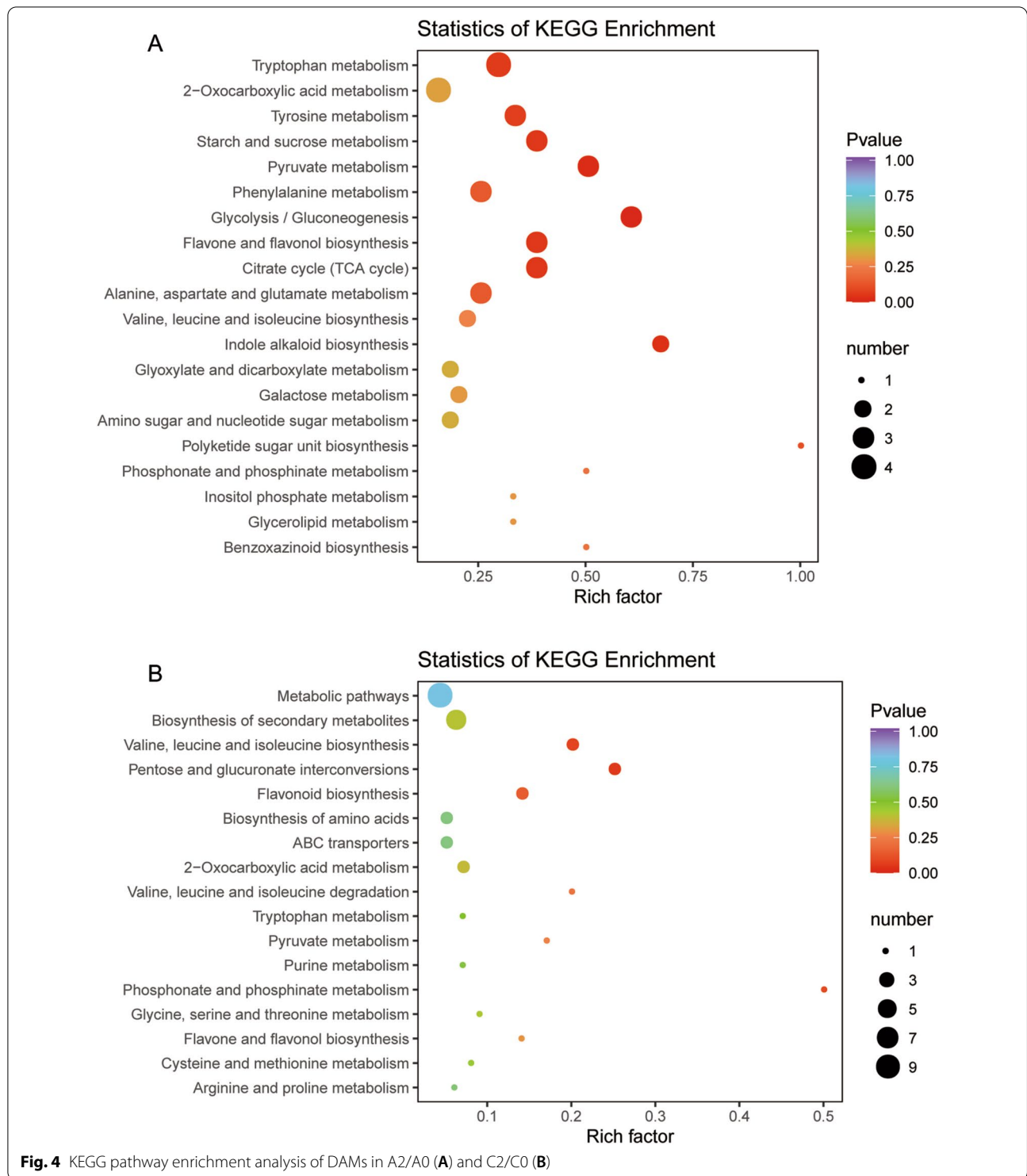


**Fig. 3** KEGG pathway enrichment analysis of DAMs in A1/A0 (A) and B1/B0 (B)

different genetic variations. In this study, 664 metabolites were identified from seeds of 7 maize lines. There were 71, 121, 43 and 95 DAMs in A1, B1, C2 and A2 GM maize seeds, respectively. These DAMs were not identified as new metabolites and showed only changes

in abundance. These results are consistent with results previously reported for other GM crops [22].

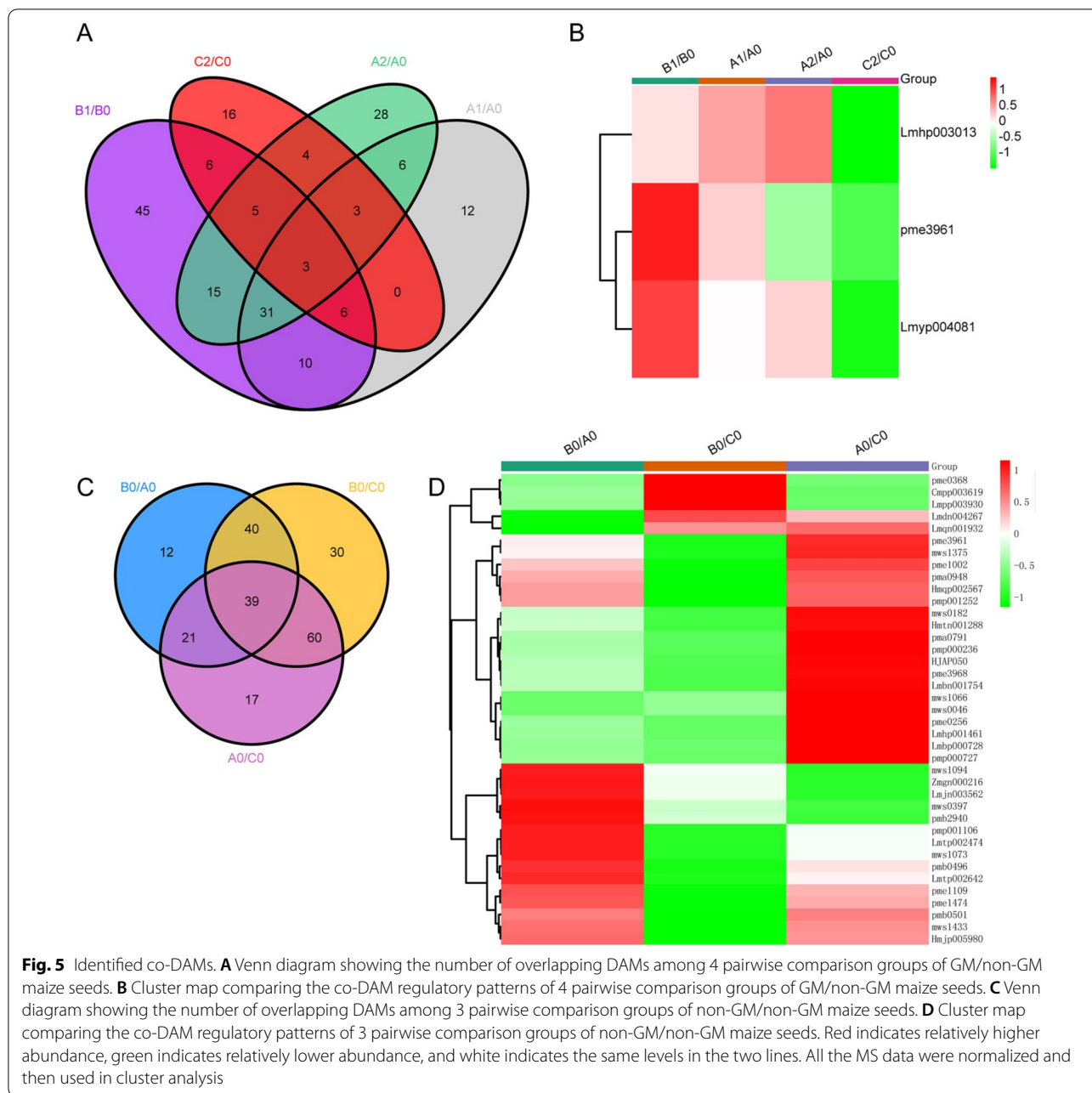
There were 112, 169 and 137 DAMs identified by comparison of the non-GM maize lines B0/A0, B0/C0 and A0/C0, respectively. The differences in metabolite



**Fig. 4** KEGG pathway enrichment analysis of DAMs in A2/A0 (A) and C2/C0 (B)

accumulation (418 DAMs and 39 co-DAMs) among the three natural genotypic maize lines were greater than those among GM/non-GM maize lines, with 330 DAMs and 3 co-DAMs observed.

Previous published proteomics data showed that the insertion of dual herbicide-resistance genes led to an increase in the number of DEPs, regardless of whether the genetic background was the same (A2/A0 compared



**Table 2** Pathways associated with co-DAMs among the 4 GM maize lines

Index	Compounds	cpd_ID	kegg_map
Lmhp003013	<i>N</i> -Feruloyl-cadaverine	–	–
pme3961	2'-Deoxyadenosine	C00559	ko00230 (purine metabolism), ko01100 (metabolic pathways)
Lmyp004081	Isovitexin-2''-O-rhamnoside	–	–

**Table 3** Number of (co)pathways involving DEPs and DAMs based on integrated proteomics and metabolomics analyses

Comparison groups	No. of pathways in which DEPs and/or DAMs are involved		
	DEPs	DAMs	Both
A1/A0	61	24	14
B1/B0	33	38	14
A2/A0	72	45	31
C2/C0	72	18	13

with A1/A0) or different (C2/C0 compared with B1/B0) [16]. However, the metabolomics data showed different regulation trends, and the insertion of dual herbicide-resistance genes (*epsps* and *pat*) led to an increase in the number of DAMs only with the same genetic background (A2/A0 compared with A1/A0); however, it led to a significant decrease in the number of DAMs with different genetic backgrounds (C2/C0 compared with B1/B0). This difference might have been due to the insertion of different foreign genes or insertions at different positions in the genome.

The difference in the number of DAMs between GM maize lines with the same exogenous genes but different receptors—A1/A0 compared with B1/B0 (71 and 121) and A2/A0 compared with C2/C0 (95 and 43)—was much higher than that between GM maize lines with identical receptor but different exogenous genes, such as A1/A0 compared with A2/A0 (71 and 95). These results showed that different genetic backgrounds affected the metabolite profiles more than gene modification itself, which was consistent with previously reported results [25].

## Conclusions

Widely targeted metabolomics was successfully used to evaluate variations in the metabolite profiles of maize seeds caused by both transgenic modifications and natural genotypic differences. The variations in metabolite accumulation among the different natural genotypic maize lines were greater than those caused by transgenic modification. The DAMs were not identified as new unintended metabolites; only their abundance changed. The different genetic backgrounds affect metabolite profiles more than gene modification itself does.

## Abbreviations

GM: Genetically modified; DAMs: Differentially accumulated metabolites; ISAAA: The International Service for the Acquisition of Agri-biotech Applications; iTRAQ: Isobaric tags with relative and absolute quantitation; *Bt*: *Bacillus thuringiensis*; EPSPS: 5-Enolpyruvate shikimic acid-3-phosphate synthase; PAT: Phosphinothricin acetyl transferase; UPLC: Ultra-performance liquid

chromatography; MS: Mass spectrum; ESI-QTRAP: Electron spray ionization-triple quadrupole-linear ion trap; QC: Quality control; OPLS-DA: Orthogonal partial least squares-discriminant analysis; VIP: The variable importance in projection; PCA: Principal component analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40538-021-00257-0>.

**Additional file 1: Figure S1.** Venn diagram showing the number of overlapping DAMs across pairwise comparisons among 4 comparison groups of GM/all non-GM maize seeds. **Figure S2.** DEPs and DAMs involved in phenylalanine, tyrosine and tryptophan biosynthesis pathways in the A2/A0 comparison group. Red dots are upregulated metabolites, green dots are downregulated metabolites, pink rectangles are upregulated proteins, and yellow rectangles are downregulated proteins. **Table S1.** List of all identified metabolites in maize seeds. **Table S2.** The list of maize DAMs in A1/A0. **Table S3.** The list of maize DAMs in B1/B0. **Table S4.** The list of maize DAMs in A2/A0. **Table S5.** The list of maize DAMs in C2/C0. **Table S6.** The list of maize DAMs in A0/C0. **Table S7.** The list of maize DAMs in B0/C0. **Table S8.** The list of maize DAMs in B0/A0.

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## Authors' contributions

WL and WJ conceived and designed the experiments. WL, MD and LM performed the experiments. WL and YW analysed the data. WL and WJ wrote the paper. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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