ORIGINAL ARTICLE

Metabolic effects of glyphosate on transgenic maize expressing a *G2-EPSPS* gene from *Pseudomonas fluorescens*

Yunjun Liu · Yuwen Zhang · Yan Liu · Wei Lu · Guoying Wang

Abstract Transgenic glyphosate-tolerant maize expressing 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene has been commercialized since 1996. However, it is not very clear how glyphosate treatment affects metabolite pathway in transgenic glyphosate-tolerant maize. Here, we obtained numerous of glyphosate-tolerant transgenic maize expressing a *Pseudomonas fluorescens G2-EPSPS* gene. The expression and integration site of *G2-EPSPS* in transgenic maize event Aro203, which can tolerate 3 folds of field usage of glyphosate, were investigated. Metabolite analysis was performed with Aro203 leaf samples using GC/MS method. The results showed that total 58 metabolites were identified. Over-expression of *G2-EPSPS* led to the increase of glutamate, malate, hydroxylamine and trehalose contents, but the decrease of glyoxylate, ribose and sucrose, compared to wild type plants. Twenty-two and 13 metabolites were up-regulated and down-regulated in non-transgenic maize by glyphosate treatments, respectively, whereas fewer metabolites (10 up-regulated and 4 down-regulated) were affected in transgenic maize. Glyphosate treatment significantly stimulated the accumulation of most amino acids but decreased lots of sugars in non-transgenic plants. The PCA analysis results showed that wild type plant cluster treated with glyphosate was clearly separated with other three clusters. The results in this study provide evidence to understand how genetic modification or glyphosate treatment affects the metabolite pathway in maize.

Keywords Maize · EPSPS · Glyphosate-tolerance · Metabolite

Abbreviations

EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
PEP	Phosphoenolpyruvic acid
S3P	3-phosphoshikimic acid
rbcS	rib-1,5-bisphospate carboxylase
GC-MS	Gas chromatography–mass spectrometry

Y. Liu · Y. Zhang · Y. Liu · G. Wang (✉)
Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Zhongguancun South Street 12, Beijing 100081, China
e-mail: gywang604@gmail.com

Y. Zhang
College of Agriculture and Biotechnology, China Agricultural University, Yuanmingyuan West Road 2, Beijing 100094, China

W. Lu
Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Zhongguancun South Street 12, Beijing 100081, China

Introduction

Glyphosate is an important herbicide and used widely to control weeds in field. Glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which convert phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid (S3P) to 5-enolpyruvylshikimate-3-phosphate (EPSP), and shuts down the shikimate pathway, leading to the plant death. The primary target of glyphosate was identified as EPSPS in the 1980s (Steinrucken and Amrhein 1980), so EPSPS was chosen as the first choice to develop transgenic glyphosate-tolerant crops. *CP4-EPSPS*, which came from *Agrobacterium* sp. strain CP4, was widely used to develop glyphosate-tolerant corn, soybean and canola due to its high Ki for glyphosate and low Km for substrate PEP (Padgette et al. 1995). Glyphosate-tolerant soybean was commercialized in 1996 and herbicide-tolerant crops occupied

234

J. Plant Biochem. Biotechnol. (April–June 2015) 24(2):233–241

89.3 million hectares of the global biotech area of 148 million hectares (James 2010), of these most herbicide-tolerant crops are tolerant to glyphosate.

Mutagenesis of EPSPS is one way to obtain glyphosate-tolerant *EPSPS* genes. Mutagenesis and structure analysis revealed the mechanism of EPSPS function (Funke et al. 2006, 2009; Schonbrunn et al. 2001). A single Ala residue at position 100 makes the CP4 EPSPS insensitive to glyphosate, whereas natural plant and bacterial enzymes share a highly conserved Gly residue at this position (Funke et al. 2006). Double mutation (P102I, P106S) of type I EPSPS cause the shift of Gly residue at positon 96 toward the glyphosate binding site, leading to the glyphosate resistance (Funke et al. 2009). A P106L mutant of rice EPSPS, which showed high glyphosate tolerance in *E.coli* and transgenic tobacco plants, was selected based on the directed evolution strategy and (Zhou et al. 2006). The mutated maize EPSPS which has two codons changed also had higher glyphosate tolerance than the original protein (Howe et al. 2002). Glyphosate-resistant EPSPS could also be obtained by screening the bacteria grown in the glyphosate-contamination environment. Recently, a glyphosate-resistant *G2-EPSPS* was cloned from bacteria and showed high glyphosate tolerance in transgenic tobacco plants (Dun et al. 2007).

Glyphosate treatment inhibits the shikimate pathway in plants, leading to the increase of shikimate and the reduction of aromatic amino acids (tryptophan, tyrosine and phenylalanine). When glyphosate-sensitive soybean plants were treated with glyphosate, up to 311 genes were identified as having different transcript levels at 24 h post-treatment. However, for the glyphosate-tolerant soybean plants expressing *CP4-EPSPS*, transcripts were marginally affected at 1 h after treatment and then quickly adjusted to glyphosate treatment (Zhu et al. 2008). Nitrogen metabolism and seed composition were influenced by glyphosate application in glyphosate-resistant soybean (Bellaloui et al. 2008). Glyphosate drift in the field caused a significant decrease of Fe concentration in glyphosate-tolerant soybean seeds and leaves (Bellaloui et al. 2009). However, how glyphosate application affects the metabolite in plants is not fully understood.

In this study, we transformed a *G2-EPSPS* gene from *Pseudomonas fluorescens* (Dun et al. 2007) which has been shown as a good candidate gene for development of glyphoste-tolerant crops, into maize HiII genotype using *Agrobacterium*-mediated transformation method. The expression, segregation and integration of *G2-EPSPS* in transgenic maize were investigated. We also investigate how glyphosate application affects the metabolites in non-transgenic and transgenic maize plants.

Materials and methods

Plasmid construction

A 283 bp fragment (part of maize ubiquitin promoter, GenBank No. S94464) was amplified using the upstream primer 5′-ATTAATTAGTGAGAAAGCTTGCATG CCTG CAGTGCAGCGT-3′ and downstream primer 5′-GGTCAC CAACTTC AGTGCACATTCCTGAGCTCACCATGTCT AACTGTTCATT-3′, adding *Ase*I and *Hind*III site at the 5′ region and *Sac*I and *Bst*EII site at the 3′ region, respectively. This fragment was digested with *Ase*I and *Bst*EII, and replaced the T-DNA region of plasmid pCAMBIA3301 digested with the same enzymes, to construct the plasmid pM3301PCR.

The 177 bp signal peptide sequence of pea *rbcS* small subunit gene (X00806) was amplified using primers 5′-TCTA GAATGGCTTCTATGATATCCTC-3′ and 5′-GGATCCGC ACTTTACTCTTCCACCAT-3′, adding *Xba*I site at the 5′ region and *Bam*HI at the 3′ region, respectively. *RbcS* fragment was cloned into plasmid pUC19, and G2 aroA fragment digested with *Bam*HI and *Sac*I was fused to the downstream of *rbcS* with the same reading frame. The fragment of ubiquitin promoter was digested from plasmid pAHC17 using *Hind*III and *Pst*I, and ligated to the upstream of *rbcs-G2 aroA*. The ubiquitin-rbcs-G2 fragment digested with *Hind*III and *Bst*EII was ligated to the plasmid pM3301PCR to construct the transformation vector pM3301UbiSpG2.

Maize transformation

The *A. tumefaciens* strain EHA105 containing the binary vector pM3301UbiSpG2 was used for transformation. Donor plants for immature embryos were HiII. Immature embryos with 1.0–2.0 mm length were isolated and suspended in liquid infection medium [MS basal medium, 68.5 g L^{-1} sucrose, 36.0 g L^{-1} glucose, 100 μM acetosyringone, pH5.2] and washed twice with this medium. The final wash was moved to an *A. tumefaciens* suspension and incubated for 5 min. Following inoculation, the embryos were transferred to solid co-cultivation medium [MS basal medium, 20 g L^{-1} sucrose, 10 g L^{-1} glucose, 0.85 mg L^{-1} silver nitrate, 100 μM acetosyringone, 8 g L^{-1} agar, pH 5.8] and incubated in the dark at 23 °C for 3 days, after which embryos were transferred to 28 °C on resting medium [N6 basal medium, 20 g L^{-1} sucrose, 10 g L^{-1} glucose, 0.85 mg L^{-1} silver nitrate, 250 mg L^{-1} cefotaxime, 8 g L^{-1} agar, pH 5.8]. After 7 days on resting medium (28 °C, dark), embryos were moved to selection medium, which was identical to resting medium with the addition of 0.5 mM glyphosate, and maintained for 2 weeks in the dark at 28 °C, and then selection was increased to 1 mM and 3 mM glyphosate for 2 round of 2-week selection, respectively. Resistant calli were placed on MS medium containing 0.01 mM glyphosate for regeneration.

The 2–3 cm shoots were moved to 1/2 MS rooting medium. Calli for regeneration and regenerated plantlets were grown in a growth chamber at 28 °C under fluorescent white light in 16/8 h light:dark cycle.

Western blot analysis

About 0.1 g leaf samples were ground in liquid nitrogen and total leaf proteins were extracted in 1 ml of $1 \times$ SDS-PAGE sample buffer. Around 20 µg protein was resolved by one-dimensional SDS-PAGE on 10 % gels, and proteins were wet-blotted to polyvinylidene difluoride membranes with transfer buffer [25 mM Tris, 10 mM glycine and 10 % (v/v) methanol, pH 8.3]. G2-EPSPS was detected with monoclonal antibody specific for G2-EPSPS protein. Heat shock protein 90 (HSP) was detected with multiclonal antibodies. Antibody recognition was visualized using mouse or horseradish peroxidase conjugated secondary antibodies.

Tail-PCR analysis

Tail-PCR was performed as described by Liu and Chen (2007) except that only two round of PCR reaction was run in this experiment. 20 µl pre-amplification reaction mixture contains 2.0 µl PCR buffer, 200 µM dNTPs, 1.0 µM LAD primer, 0.3 µM RB-0a or RB-0b, 0.5 U Ex Taq, and 20–30 ng transgenic maize genomic DNA. Each 25-µl primary TAIL-PCR contained 2.5 µl PCR buffer, 200 µM dNTPs, 0.3 µM AC1 and RB-1a (or RB-1b), 0.6 U Ex Taq, and 1 µl 40-fold diluted pre-amplified product. The PCRs were performed using GeneAmp® PCR System 9700. The PCR products were analyzed on 1.0 % agarose gels, and specific fragments were purified using a DNA purification kit and sequenced.

Metabolite analysis

Seeds of WT and homozygous transgenic plants were sown in the soil in the pots and grown in the growth chamber at 28 °C with a 16/8 h light/dark cycle. Two-week-old plants were sprayed with 2.52 kg ha^{-1} glyphosate, and the leaf samples were taken 2 days after treatment.

The metabolite extraction and GC-MS analysis was performed as described (Roessner et al. 2000) with some modification. 500 mg leaf samples were ground in liquid nitrogen, and extracted in 4 ml methanol for 30 min, then the extract was centrifuged at 2,200 g. Aliquots of the supernatant (500 µl) were dried in vacuo. The dried residue was re-dissolved in 100 µl methoxyamine hydrochloride (20 mg/ml) for 90 min at 30 °C, then treated with 200 µl MSTFA for 30 min at 37 °C. The samples were then kept for 12 h at room temperature.

1 µl of samples were injected into GC-MS system (Thermo DSQII, USA). Gas chromatography was performed on a 30 m TR-5MS column with 0.25 mm inner diameter and 0.25 µm film thickness. Injection temperature was 200 °C. The carrier gas used was helium set at a constant flow rate of 1 ml min^{-1}. The temperature program was 5 min isothermal heating at 100 °C, followed by a 5 °C min^{-1} temperature ramp to 300 °C, then kept for 5 min. Mass spectra were recorded at 2.38 scans s^{-1} with an m/z 50–650 scanning range. Peak identification was performed by employing AMDIS (NIST, Gaithersburg, MD, USA) and WILEY7n (Palisade Corporation, NY, USA) databases with a spectral match quality >90 %. All data were normalized to total peak area.

Results

Obtainment and identification of transgenic maize

In the T-DNA region of plant transformation vector pM3301UbiSpG2 (Supplemental Fig. 1), signal peptide sequence of pea rib-1,5-bisphospate carboxylase (rbcS) small
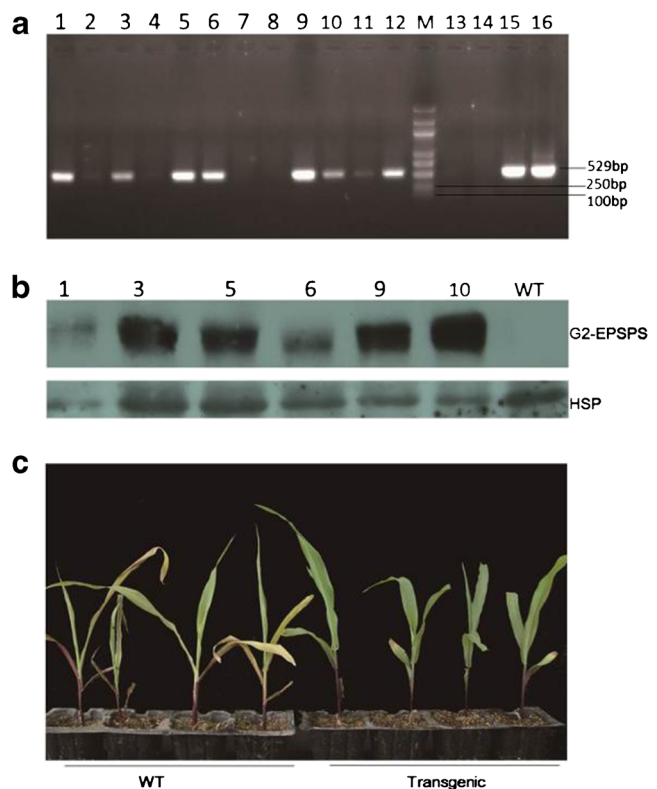
Fig. 1 Molecular identification and glyphosate-tolerance assessment of Aro203. **a** PCR amplification of G2-EPSPS gene. 1–12: T1 generation plants of Aro203; 13: H$_2$O; 14: Wild-type plant; 15–16: Plasmid pM3301UbiSpG2. **b** Western blot of transgenic plants. 1,3,5,6,9,10: PCR-positive T1 generation plants of Aro203; WT: wild-type plants as negative control. **c** The phenotype of transgenic plants 3 days after sprayed with 4 g L^{-1} glyphosate with a dose of 2.46 kg/ha

Table 1 Segregation of *G2-EPSPS* in the generation of glyphosate-tolerant transgenic maize

Generation	Pollination	Tolerant	Sensitive	Expected ratio	X^2
T1	Self crossed	21	7	3:1	0.00[*]
F1	Cross with Zong31	7	11	1:1	0.89[*]
F2	Self crossed of F1	70	28	3:1	0.67[*]

[*] Significantly deviates from expected ratio at $P=0.01$ level

subunit was fused in front of *G2-EPSPS* gene, to direct the EPSPS to the plant chloroplast, and the gene transcript was controlled with maize ubiquitin promoter. *G2-EPSPS* gene is not only the selective marker gene but also the interested gene. Vector pM3301UbiSpG2 was transformed into maize using the method described in Materials and Methods. Among the obtained independent transgenic events, one line which was named as Aro203 showed high tolerance to glyphosate and was further characterized.

Total genomic DNA was isolated from T1 generation plants of Aro203, and PCR amplification was performed with the primers specific for *G2-EPSPS* (Supplemental Table 1). A 529 bp fragment could be amplified in transgenic plants, whereas no PCR products were produced in un-transgenic plants (Fig. 1a), confirming the integration of target gene in the maize genome. Proteins were extracted from PCR-positive transgenic plant leaves and used for western blotting to investigate the expression level of *G2-EPSPS* gene. High level of G2-EPSPS protein existed in the transgenic plant leaves, however different protein amount was observed in different individual plants (Fig. 1b).

The inheritance of *G2-EPSPS* gene in different generation was investigated by spraying plants with 2.46 kg ha^{-1} glyphosate which is 3 folds of field usage (Fig. 1c). Transgenic plants grew well whereas wild-type plants began to die 3 days after treatment, indicating the tolerance of transgenic plants to glyphosate. The T1, F1 and F2 generation fitted to the expected 3:1, 1:1 and 3:1 Mendelian ratio, respectively (Table 1). These results demonstrated that the glyphosate-tolerant phenotype was inherited as a single Mendelian locus.

Characterization of the Aro203 integration site

The integration site of *G2-EPSPS* gene in Aro203 was investigated using the tail-PCR method. The tail-PCR primers were designed on the *NOS* terminator sequence as described in Experimental procedures. A 325-bp PCR product which includes *NOS* fragment and some maize genomic fragment was obtained (Fig. 2a). After sequencing this fragment and blasting in www.maizesequence.org, it was confirmed that the 3′ flanking sequence of insertion fragment of Aro203 is the sequence from 227,594,469 to 227,594,793 of maize chromosome 1. Then the primers were designed based on the sequence upstream 227,594,469 site of maize chromosome 1 and the ubiquitin promoter, and used to amplify the upstream sequence of insertion site (Fig. 2b). The sequencing results of amplified fragment confirmed that the maize sequence from 227,594,252 to 227,594,468 of chromosome 1 was obtained. So the T-DNA region of plasmid pM3301UbiSpG2 was integrated in the 227,594,468/9 site of the first chromosome of maize.

Then the specific primers ZmF, ZmR and G2F were designed according to the sequence of maize genome and *G2-EPSPS*, respectively (Fig. 3a, Supplemental Table 1). A 526 bp fragment could be amplified for the wild-type plants, and a 751 bp fragment could be amplified for the transgenic plants. If the transgenic plants were heterozygous, both PCR products could be obtained (Fig. 3b). When analyzing the F1 progeny of Aro203 using these three primers, half of plants were identified as heterozygous (Fig. 3b), confirming the PCR method for the identification of Aro203 event is feasible. This simple and fast zygosity test method could help the effective trait integrations.

Fig. 2 Determine the insertion site of T-DNA region using tail PCR. **a**: Tail PCR analysis. M, DL2000 DNA ladder(Beijing TransGen Biotech Co., Ltd., China); 1, event Aro1149; 2, WT; 3, event Aro203 **b**: Amplify upstream sequence of insertion site with normal PCR. M, DL2000 plus DNA ladder(Beijing TransGen Biotech Co., Ltd.,China); 1–2, WT; 3–4, H$_2$O; 5, event Aro203
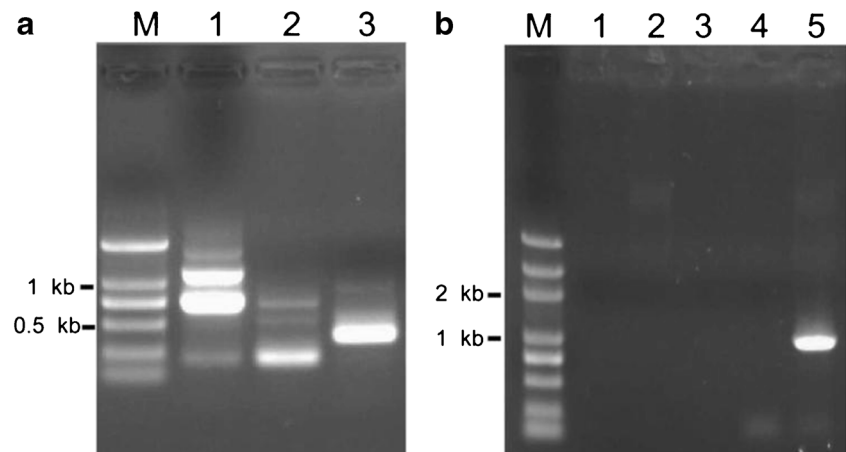
Fig. 3 Aro203 zygosity test. **a**, Schematic diagram of Aro203 transgenic locus. **b**, PCR amplification of Aro203 progeny using three primers shown in **a**. 1–8, F1 progeny of Aro203 event; 9, H$_2$O; 10–11, WT; 12, Plasmid pM3301UbiSpG2; M, DL2000 plus DNA ladder(Beijing TransGen Biotech Co., Ltd., China)
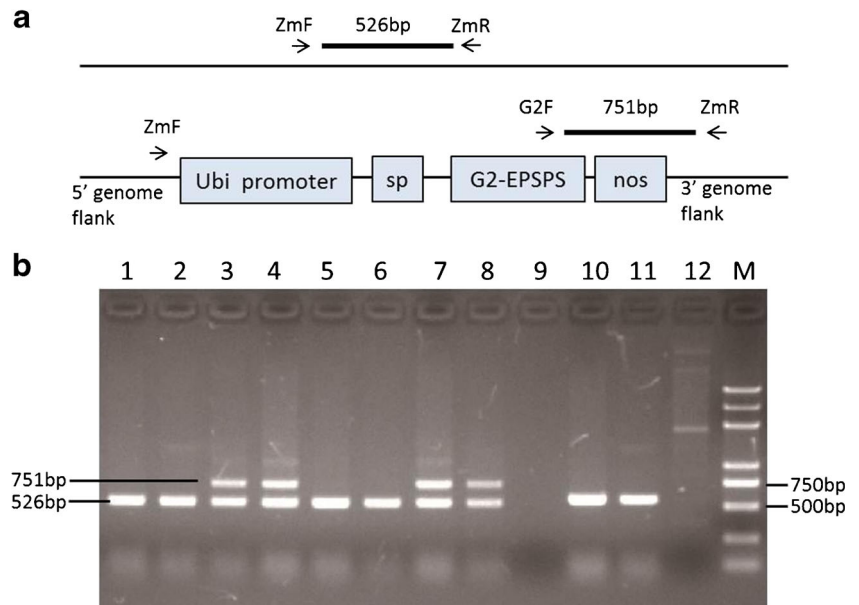

Metabolite analysis of transgenic and non-transgenic maize

To further investigate how the over-expression of *EPSPS* in transgenic maize affects the response of transgenic maize to glyphosate treatment, we performed GC-MS analysis on the WT and transgenic plants, and total 58 metabolites were detected (Supplemental Table 2). The over-expression of *G2-EPSPS* in transgenic maize led to the increase of glutamate, malate, hydroxylamine and trehalose contents, but the decrease of glyoxylate, ribose and sucrose, compared to WT plants (Figs. 4 and 5). When WT plants were treated with 2.46 kg ha^{-1} glyphosate, 22 metabolites were up-regulated

and 13 metabolites were down-regulated (Figs. 4 and 5). Most detected amino acids in WT plants were up-regulated by glyphosate treatment, meanwhile the contents of most detected sugars were decreased except for an increase of sucrose content. Glyphosate shut down the shikimate pathway by inhibiting the EPSPS which convert PEP and S3P to EPSP. Glyphosate treatment led to a 16 folds increase of shikimate content in WT plants, however shikimate contents in transgenic maize was not affected by glyphosate application, indicating the glyphosate-tolerance of transgenic maize. Compared to the highly affected metabolites by glyphosate treatment in WT plants, fewer metabolites in transgenic maize

Fig. 4 Number of metabolites affected by over-expression of *G2-EPSPS* or by glyphosate treatment

238

J. Plant Biochem. Biotechnol. (April–June 2015) 24(2):233–241
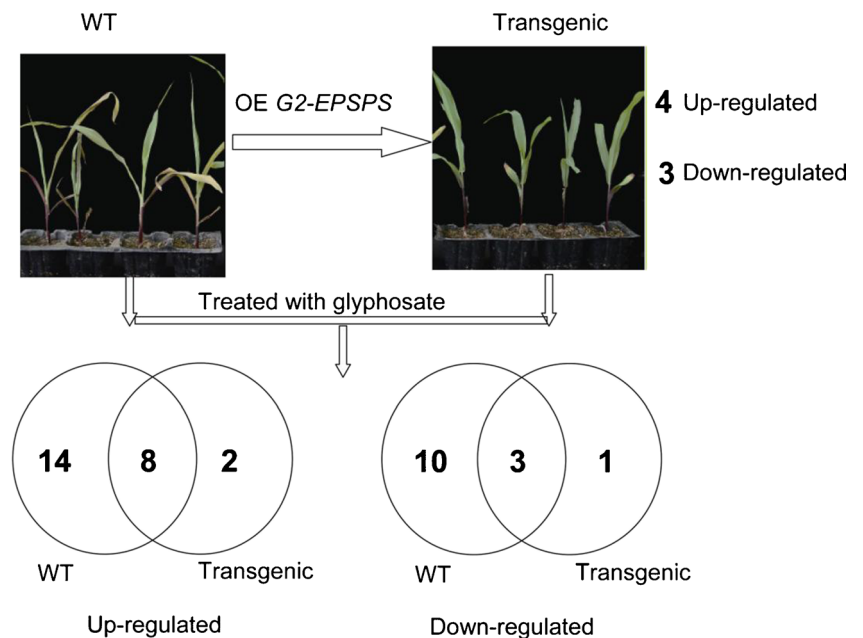
Fig. 5 Metabolic changes in maize leaves. **a**, The metabolic difference between WT and transgenic maize plants. **b**, Metabolic changes following treatment of WT maize plants with glyphosate. Data are presented with average ± S.E. of six independent replicates
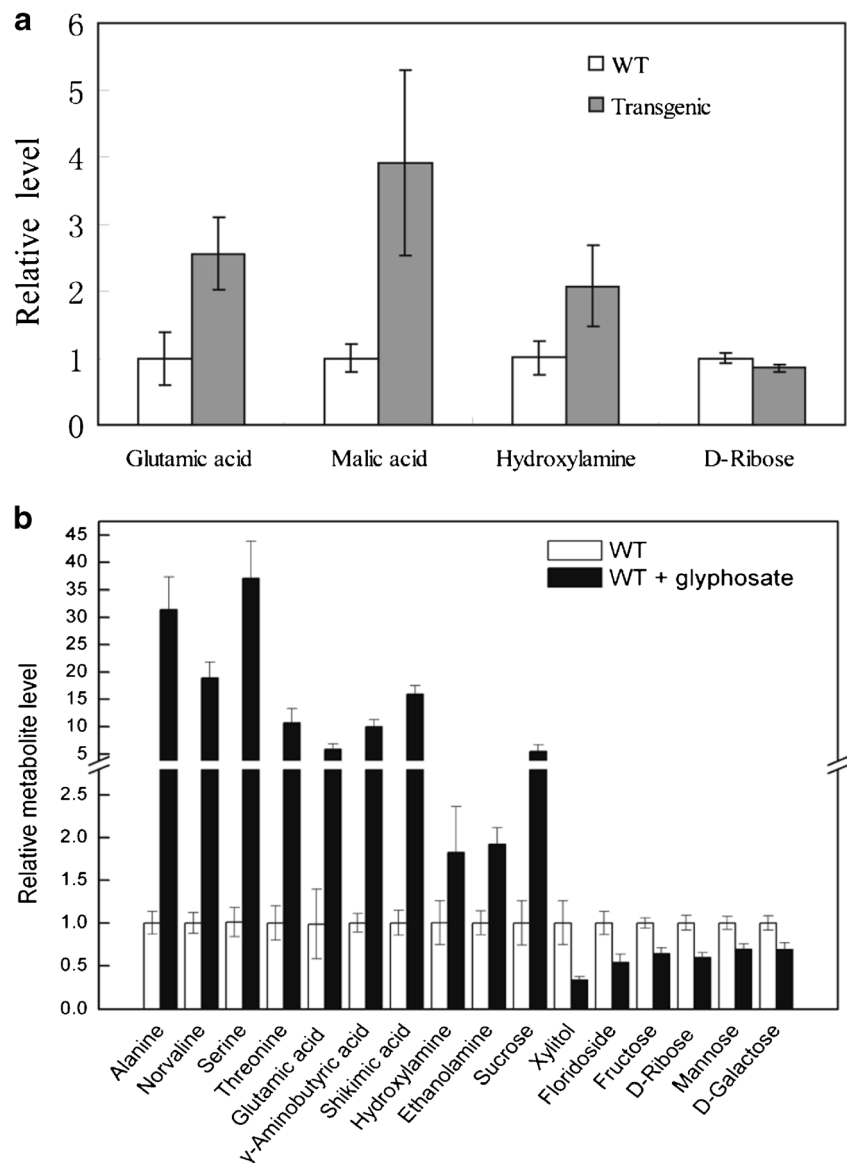

responded to glyphosate treatment with 10 upregulated metabolites and 4 down-regulated metabolites, respectively. Glyphosate treatment did not affect the sugar contents in transgenic maize plants. Several amino acids (alanine, aspartate, asparagines, serine and glycine) in transgenic plants showed same up-regulated pattern as that in WT plants.

Metabolites were assigned to metabolic pathway based on KEGG analysis, and it clearly showed that WT and transgenic maize responded differently to the glyphosate treatment (Fig. 6). To further analyze the data about metabolite change, we performed PCA analysis. The PCA results showed that WT treated with glyphosate was clearly separated with other three clusters. Glyphosate treatment had a large effect on WT plants and less effect on transgenic plants, and over-expression of *G2-EPSPS* had less impact on plants (Fig. 7).

Discussion

G2-EPSPS gene has been cloned from *Pseudomonas fluorescens* and the transgenic tobacco plants overexpressing *G2-EPSPS* showed good performance of glyphosate-tolerance (Dun et al. 2007). Here, we transformed *G2-EPSPS* gene into maize to investigate whether it can confer high

Fig. 6 Metabolic pathway affected by over-expression of *G2-EPSPS* or ▶ by glyphosate treatment. *Upper triangle* indicate change in WT treated with glyphosate compared to WT; *Lower triangle* indicated change in transgenic plants treated with or without glyphosate; *rectangle* indicate change in transgenic plants compared to WT; *Gray color* indicate no difference; *Red color* indicate up-regulated; *Green color* indicate down-regulated; Metabolites in *red* indicate the detected in this study; *solid line* indicate no intermediate; *dash line* indicate that there are intermediates between two metabolites
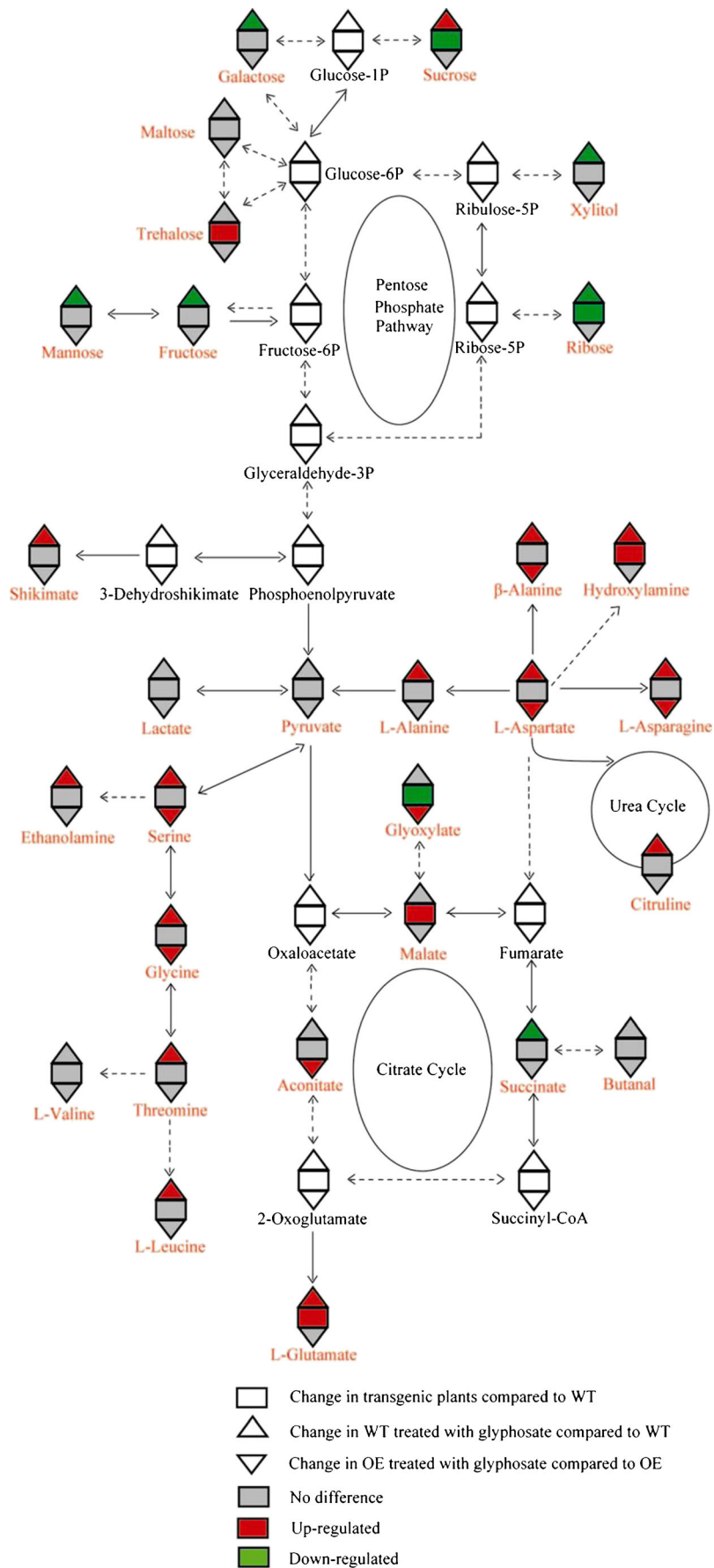
Fig. 7 PCA of metabolites in WT and transgenic plants treated with or without glyphosate

△ WT
▲ WT treated with glyphosate
□ Transgenic
■ Transgenic treated with glyphosate
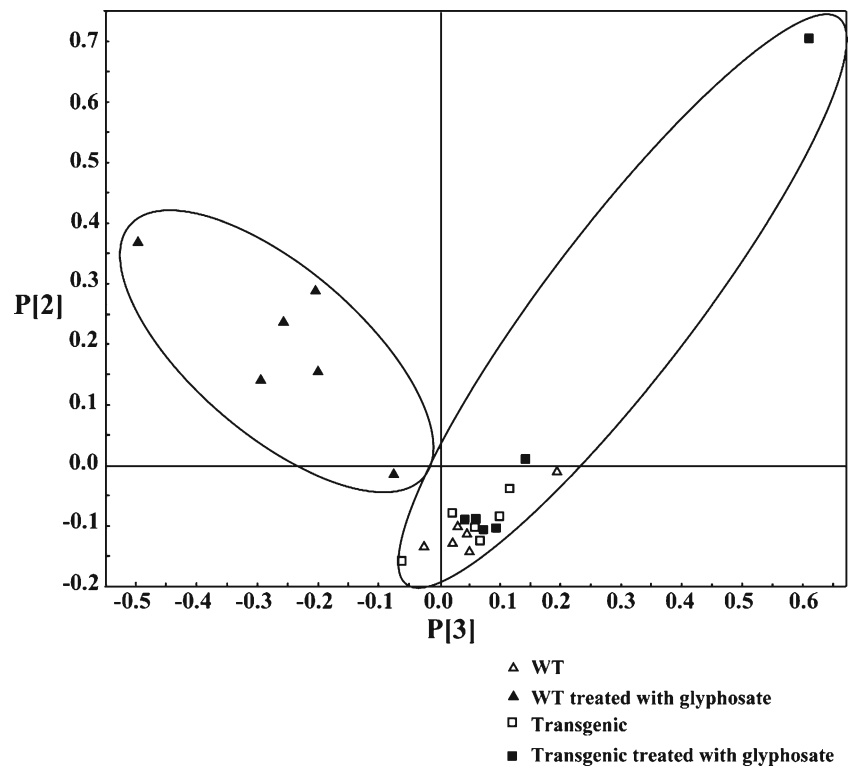
concentration of glyphosate. We constructed a plant transformation vector pM3301UbiSpG2 (Supplemental Fig. 1) where *G2-EPSPS* gene is used as not only selectable marker gene also as an candidate gene conferring glyphosate tolerance. We did see the negative effect of glyphosate on the plant regeneration (data not shown). More than one hundred transgenic events were obtained, demonstrating that *G2-EPSPS* gene can be used as a selectable marker gene for the obtainment of transgenic maize plants. The glyphosate-tolerance of these transgenic events was assessed by spraying glyphosate in the field and several events could tolerate 3 fold of glyphosate usage in the field. One of them, Aro203, which has a Mendelian inheritance for a single insertion site, was further analyzed.

This Aro203 event could be integrated into local elite corn inbred lines through backcrossing coupled with application of glyphosate for selection. Gene-specific, construct-specific and event-specific PCR strategies have been used to detect transgenic plants, however they have different ability to discriminate between transgenic and non-transgenic plants (Yang et al. 2007). Event PCR with the primers specific for the insertion site could accurately identify the transgenic plants and fasten the identification of plant zygosity. Tail-PCR is an effective method to isolate the T-DNA insertion flanking sequence (Liu and Chen 2007), and has been successfully used to isolate the flanking sequence of lots of transgenic events (Huang and Pan 2004; Yang et al. 2007). 5′ flanking genomic DNA sequence of Aro203 was obtained using tail PCR and the 3′ flanking genomic DNA sequence was obtained using normal PCR

(Fig. 2). The PCR reaction was performed on the F1 progeny with three primers designed based on the 5′ and 3′ flanking genomic sequence and *G2-EPSPS* sequence, and the zygosity and negative plants could be distinguished.

Application of glyphosate could shut down the shikimate pathway, leading to the accumulation of shikimate and some hydroxybenzoic acids, and reducing the biosynthesis of aromatic amino acids and some secondary compounds (Hernandez et al. 1999). Several reports have shown that glyphosate can also affect nitrogen metabolism and seed composition (Bellaloui et al. 2009; Reddy et al. 2010). However, metabolic alterations caused by glyphosate application in plants, especially in maize, are not fully understood. The suitability of GC/MS for the identification of metabolites has been used in maize (Barros et al. 2010). In this study, we investigated the influence of genetic modification or the glyphosate application on the maize plants.

First, we evaluated the consequences of genetic modification (transgenic plants versus non-transgenic plants). Among the identified 58 metabolites, over-expression of *G2-EPSPS* only increased four metabolites (glutamate, malate, hydroxylamine and trehalose) and reduced three metabolites (glyoxylate, ribose and sucrose) with small change folds (Figs. 5 and 6 and supplemental Table 2). These data indicates that genetic modification did not lead to numerous metabolite change, which is useful for food safety assessment. In the transgenic glyphosate-tolerant soybean, three metabolites (liquiritigenin 6-coumaroylglucoside, naringenin 7-O-

glucoside and 6-methoxytaxifolin) were elevated, while five metabolites (proline, histidine, asparagines, gluconic acid and trihydroxypentanoic acid) were reduced (Garcia-Villalba et al. 2008). The results of ours and Garcia-Villalba et al.(2008) clearly showed that over-expression of EPSPS did not lead to the change of metabolites.

Twenty-two and 13 metabolites were up-regulated and down-regulated in non-transgenic maize by glyphosate treatments, respectively, whereas fewer metabolites (10 up-regulated and 4 down-regulated) were affected in transgenic maize, meaning transgenic maize could more tolerate glyphosate treatment than non-transgenic maize plants. Application of glyphosate led to a dramatic accumulation (16 folds) of shikimate in non-transgenic maize plants, whereas the shikimate content in the transgenic maize over-expressing *G2-EPSPS* was not affected, which is consistent with the previous reports where non-glyphosate-resistant soybean accumulated much higher shikimate than glyphosate-resistant soybean (Bellaloui et al. 2008). High accumulation (above 10 folds) of alanine, norvaline, serine, threonine and aminobutyric acid (GABA) were also observed in non-transgenic maize. Most detected amino acids were elevated and sugars were decreased in glyphosate-treated non-transgenic maize, which is consistent with the results from glyphosate-treated maize callus (Ulanov et al. 2009). Aspartic acid and asparagines, two main amino acids engaged in nitrogen assimilation, were elevated both in transgenic and non-transgenic maize by glyphosate supply. It has been reported that glyphosate application affected numerous of transcripts and miRNA in wheat (Unver et al. 2010). Glyphosate might affect other more metabolites which we did not detect currently.

References

Barros E, Lezar S, Anttonen MJ, van Dijk JP, Rohlig RM, Kok EJ, Engel KH (2010) Comparison of two GM maize varieties with a near-isogenic non-GM variety using transcriptomics, proteomics and metabolomics. Plant Biotechnol J 8:436–451

Bellaloui N, Zablotowicz RM, Reddy KN, Abel CA (2008) Nitrogen metabolism and seed composition as influenced by glyphosate application in glyphosate-resistant soybean. J Agric Food Chem 56:2765–2772

Bellaloui N, Reddy KN, Zablotowicz RM, Abbas HK, Abel CA (2009) Effects of glyphosate application on seed iron and root ferric (III) reductase in soybean cultivars. J Agric Food Chem 57:9569–9574

Dun BQ, Wang XJ, Lu W, Zhao ZL, Hou SN, Zhang BM, Li GY, Evans TC Jr, Xu MQ, Lin M (2007) Reconstitution of glyphosate resistance from a split 5-enolpyruvyl shikimate-3-phosphate synthase gene in *Escherichia coli* and transgenic tobacco. Appl Environ Microbiol 73:7997–8000

Funke T, Han H, Healy-Fried ML, Fischer M, Schonbrunn E (2006) Molecular basis for the herbicide resistance of Roundup Ready crops. Proc Natl Acad Sci U S A 103:13010–13015

Funke T, Yang Y, Han H, Healy-Fried M, Olesen S, Becker A, Schonbrunn E (2009) Structural basis of glyphosate resistance resulting from the double mutation Thr97 ->Ile and Pro101 ->Ser in 5-enolpyruvylshikimate-3-phosphate synthase from *Escherichia coli*. J Biol Chem 284:9854–9860

Garcia-Villalba R, Leon C, Dinelli G, Segura-Carretero A, Fernandez-Gutierrez A, Garcia-Canas V, Cifuentes A (2008) Comparative metabolomic study of transgenic versus conventional soybean using capillary electrophoresis-time-of-flight mass spectrometry. J Chromatogr A 1195:164–173

Hernandez A, Garcia-Plazaola JI, Becerril JM (1999) Glyphosate effects on phenolic metabolism of nodulated soybean (*Glycine max* L. merr.). J Agric Food Chem 47:2920–2925

Howe A, Gasser C, Brown S, Padgette S, Hart J, Parker G, Fromm M, Armstrong C (2002) Glyphosate as a selective agent for the production of fertile transgenic maize (*zea mays*) plants. Mol Breeding 10:153–164

Huang HY, Pan TM (2004) Detection of genetically modified maize MON810 and NK603 by multiplex and real-time polymerase chain reaction methods. J Agric Food Chem 52:3264–3268

James C (2010) Global status of commercialized biotech/GM crops: ISAAA Brief. ISAAA, Ithaca

Liu YG, Chen Y (2007) High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. Biotechniques 43:649–650, 652, 654 passim

Padgette S, Kolacz K, Delannay X, Re D, Lavallee B, Tinius C, Rhodes W, Otero Y, Barry G, Eichholtz D, Peschke V, Nida D, Taylor N, Kishore G (1995) Development, identification, and characterization of a glyphosate-tolerant soybeans line. Crop Sci 35:1451–1461

Reddy KN, Bellaloui N, Zablotowicz RM (2010) Glyphosate effect on shikimate, nitrate reductase activity, yield, and seed composition in corn. J Agric Food Chem 58:3646–3650

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry. Plant J 23:131–142

Schonbrunn E, Eschenburg S, Shuttleworth WA, Schloss JV, Amrhein N, Evans JN, Kabsch W (2001) Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. Proc Natl Acad Sci U S A 98:1376–1380

Steinrucken HC, Amrhein N (1980) The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. Biochem Biophys Res Commun 94:1207–1212

Ulanov A, Lygin A, Duncan D, Widholm J, Lozovaya V (2009) Metabolic effects of glyphosate change the capacity of maize culture to regenerate plants. J Plant Physiol 166:978–987

Unver T, Bakar M, Shearman RC, Budak H (2010) Genome-wide profiling and analysis of Festuca arundinacea miRNAs and transcriptomes in response to foliar glyphosate application. Mol Genet Genomics 283:397–413

Yang L, Guo J, Pan A, Zhang H, Zhang K, Wang Z, Zhang D (2007) Event-specific quantitative detection of nine genetically modified maizes using one novel standard reference molecule. J Agric Food Chem 55:15–24

Zhou M, Xu H, Wei X, Ye Z, Wei L, Gong W, Wang Y, Zhu Z (2006) Identification of a glyphosate-resistant mutant of rice 5-enolpyruvylshikimate 3-phosphate synthase using a directed evolution strategy. Plant Physiol 140:184–195

Zhu J, Patzoldt WL, Shealy RT, Vodkin LO, Clough SJ, Tranel PJ (2008) Transcriptome response to glyphosate in sensitive and resistant soybean. J Agric Food Chem 56:6355–6363