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Transcriptome and nutritional composition analysis of stacked transgenic maize with insect resistance and herbicide tolerance

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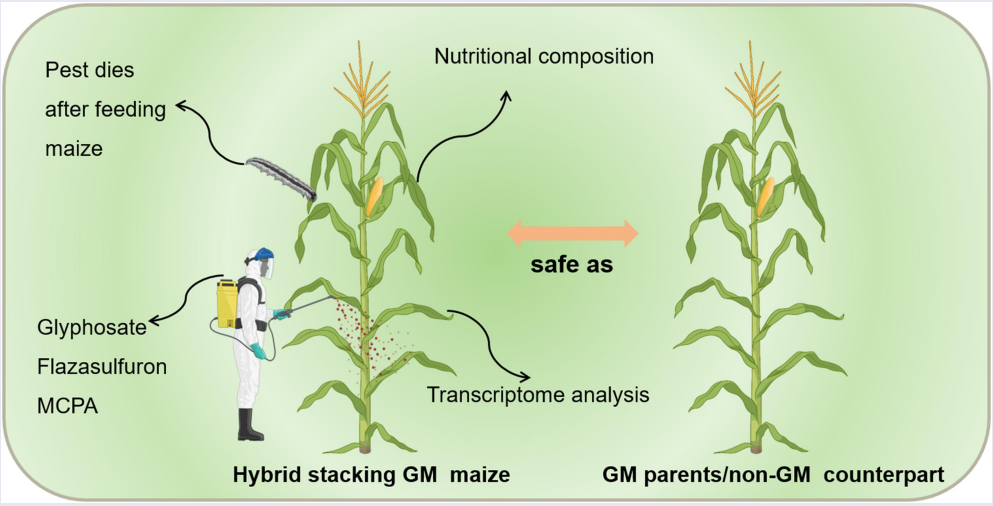
ABSTRACT

The safety assessment of stacked transgenic crops is essential for their commercial cultivation. A crucial element of safety assessment is the nutritional evaluation of transgenic crops. Currently, profiling methods like transcriptome are employed as supplemental analytical tools to find the unintended effects of transgenic crops. In this study, stacked transgenic maize ZDRF8×nCX-1 was produced by crossing of two transgenic maize events ZDRF8 and nCX-1. This stacked transgenic maize expresses five genes: *cry1Ab*, *cry2Ab* and *g10evo-epsps* (from ZDRF8), as well as *cp4 epsps* and *P450-N-Z1* (from nCX-1). Molecular analysis showed that the insertion sites of target genes were not changed during stack breeding, and the target genes are effectively expressed at both RNA and protein levels in ZDRF8×nCX-1. Target trait analysis showed that ZDRF8×nCX-1 exhibits tolerant to glyphosate, flazasulfuron and MCPA, and is resistant to damage by corn borers. Transcriptome analysis revealed that gene-stacked maize ZDRF8×nCX-1 did not significantly alter transcriptome profiles compared to the transgenic maize events ZDRF8 and nCX-1. Nutritional composition analysis showed that the grain profile of ZDRF8×nCX-1 was substantially equivalent to that of the non-transgenic counterpart. These results suggest that hybrid stacking does not cause significantly unintended effects beyond providing the intended beneficial traits.



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Introduction

Biotechnology enables the precise introduction of favorable traits through genetic modification, overcoming limitations of traditional breeding methods.[1] These advancements can be integrated with conventional breeding methods to produce hybrid crops that offer combined benefits, such as enhanced productivity, resilience, and sustainability, which is critical for addressing global food security and environmental challenges.[2–4] For example, stacked-trait crops can simultaneously address weed and pest challenges, improving

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agronomic performance. In recent years, there has been a significant rise in the development and release of stacked-trait products, including those incorporating two or more genetically modified (GM) traits.[5] From 1996 to 2023, 3176 GM crop events were approved globally, with 1436 being stacked-traits events (http://jsppa.com.cn/news/jingji/13128.html). However, their widespread adoption has led to discussions about their safety compared to single-trait GM crops or non-GM crops.

Regulatory frameworks address these concerns by requiring rigorous safety assessments.[6] Individual traits in stacked-trait crops are typically approved separately before being combined. This means that each trait has already undergone extensive safety testing. For example, if a stacked-trait crop combines herbicide tolerance and insect resistance, both traits would have been independently assessed for safety prior to being stacked. A key focus with stacked-trait crops is evaluating potential interactions between traits, such as altered gene expression or unintended compositional changes. Regulatory agencies mandate comparative safety assessments using the principle of substantial equivalence, where stacked-trait crops are compared to non-GM counterparts to ensure no significant differences in nutrition.[7] If deemed equivalent, they are considered as safe as conventional crops.

Compositional analysis is an important aspect to be assessed in the determination of substantial equivalence.[8] The Organization for Economic Cooperation and Development (OECD) guidelines have stated that the comparison of composition should be between the modified varieties and non-modified counterpart with an appropriate history of safe use, in which the data for the non-modified counterpart can be the natural ranges published in the literature for commercial varieties or other edible varieties of the species.[9] Crucially, compositional data from GM parents cannot be extrapolated to stacked event due to potential hybrid breeding effects.[10] Therefore, compositional analysis of the GM stacking events is crucial for risk assessment to determine whether any potential adverse effects may arise from a change in composition. With the development of omics techniques, transcriptome analysis has been applied to identify unintended effects in GM crops, including GM rice,[11–14] soybean,[15,16] maize,[17–19] and canola.[20] For stacked GM crops, Wang et al.[11,19] reported that hybrid stacking has less impact on gene expression than genetic modification and different crop varieties, and no new safety concerns regarding gene-stacked events have been raised.

In this study, we present the results of the safety assessment of a gene-stacked maize product. By comparing the nutritional composition values of the stacked transgenic maize ZDRF8×nCX-1 with its non-transgenic counterpart and using transcriptomic analysis to evaluate the potential safety risks from unintended effects, we found that the composition of ZDRF8×nCX-1 maize grain is substantially equivalent to that of its non-transgenic comparator, hybrid stacking caused no detrimental effects. Also, there is no evidence to suggest that they pose greater risks than single-trait GM crops or non-GM crops when properly regulated. Nonetheless, continuous monitoring, transparent communication, and robust regulatory oversight remain essential to address emerging concerns and foster public trust in GM technologies.

Materials and Methods

Plant Materials

Three transgenic maize lines (ZDRF8, nCX-1, and ZDRF8×nCX-1) and their isogenic control maize, Ruifeng-1 (RF1), were used in this study. The transgenic maize ZhedaRuifeng8 (ZDRF8) contains *cry1Ab*, *cry2Ab* and *g10evo-epsps* (a selection marker) gene expression cassettes, conferring resistance to lepidopteran pests and tolerance to glyphosate. The transgenic maize nCX-1 contains *P450-N-Z1* and *cp4 epsps* gene expression cassettes, providing tolerance to flazasulfuron, MCPA and glyphosate. The stacked transgenic maize ZDRF8×nCX-1 was developed by crossing the transgenic maize ZDRF8 and nCX-1 (Figure S1). The F1 generation was produced using ZDRF8 as the maternal parent and nCX-1 as the paternal parent. Homozygotes containing all exogenous genes from ZDRF8 and nCX-1 were obtained through two successive backcrosses with nCX-1, followed by three rounds of self-crossing. Additionally, ZDRF8×nCX-1 and RF1 were

crossed to generate ZDRF8×nCX-1-F1 generation. Ruifeng-1, the non-transgenic maize used as a control, was provided by Ruifeng Biotechnology Co., Ltd., Hangzhou.

Genomic DNA Extraction and Event-Specific PCR Amplification

Genomic DNA was extracted from approximately 100 mg of leaf tissue using a modified CTAB method.[21] For event-specific PCR detection of ZDRF8, primer pairs ZDRF8-LB-F1 and ZDRF8-LB-R1 were designed based on the left flanking sequences of the maize genome and the left border of the T-DNA. Similarly, for event-specific PCR detection of nCX-1, primer pairs nCX-1-RB-F1 and nCX-1-RB-R1 were designed based on the right flanking sequences of the maize genome and the right border of the T-DNA. The sequences of the primers used and the sizes of the amplified DNA fragments are listed in Table 1.

Target Gene Expression Analysis of Transgenic Maize

Total RNA was extracted using the Easy Plant RNA Kit (DR040650, YSD-Bio, Hangzhou, China). The cDNA was synthesized using HiScript II Q RT SuperMix for qPCR Kit with gDNA wiper (R223, Vazyme, Nanjing, China), following the manufacturer's protocols. RT-PCR was conducted in a total volume of 20 µL with the following reaction cycles: 3 min at 95°C, followed by 33 cycles of 95°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec, with a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on a 2% agarose gel. The sequences of the primers used and the sizes of the amplified cDNA fragments are listed in Table 2.

Table 2. Primers used for RT-PCR in this study.

Target fragment	Primer	Sequence (5′- 3′)	Size (bp)
cry1Ab	cry1Ab-RT-F	GCTGGACATCGTGAGCCTGTTC	207
	cry1Ab-RT-R	GCGTCGGTGTAGATGGTGATGC	
cry2Ab	cry2Ab-RT-F	GCACAACCGCAAGAACAACATCC	271
	cry2Ab-RT-R	GATGGTGGAGTTGCCGATGGAAG	
g10evo-epsps	G10-RT-F	CGCTCAGCCATCCAAGAACTACAC	285
	G10-RT-R	GTCACGAAAGTGGTGCCAGAGG	
cp4 epsps	CP4-RT-F	GGCGAAGGCTGAGATGCTACAC	240
	CP4-RT-R	GTGTCACCTTCCTTGCGGATACG	
P450-N-Z1	N-Z1-RT-F	TCATTGCTATGCTGCTCACTCTGC	227
	N-Z1-RT-R	CATGTCGTCGGCGGTAAGAAGG	
zSSIIb	zSSIIb-RT-F	CTCCCAATCCTTTGACATCTGC	151
	zSSIIb-RT-R	TCGATTTCTCTCTTGGTGACAGG	

ELISA (Enzyme-Linked Immunosorbent Assay)

The contents of Cry1Ab, Cry2Ab, G10evo-EPSPS, CP4 EPSPS and P450-N-Z1 were assessed using ELISA kits (Cry1Ab, AA0341, YouLong, China; Cry2Ab, Cropedit, China; G10evo-EPSPS, AA1141, YouLong, China; CP4 EPSPS, AA0841, YouLong, China; P450-N-Z1, ZD-EF-010, Zoonbio, China). Eight biological replicates of leaves at the 7–8 leaf stage were performed for different maize lines.

Identification of Target Traits in Transgenic Maize

For herbicide tolerance detection, maize plants at the 3–4 leaf stage were treated with glyphosate (1800 g of acid equivalent [ae] ha^{-1}) and flazasulfuron (22.5 g of active ingredient [ai] ha^{-1}. Plant herbicide tolerance was analyzed 10 days after glyphosate and flazasulfuron application.

A bioassay method was used to evaluate the pest resistance of transgenic maize and non-transgenic maize. The bioassay was performed with five replicates for each maize type. For each replicate, 20 corn borers were inoculated into a culture plate containing young maize leaves. The culture plates were sealed with parafilm to prevent larvae from escaping and then placed in a climate-controlled room with the following conditions: 28°C, 80% relative humidity,

Table 1. Primers used for event-specific PCR in this study.

Target fragment	Primer	Sequence (5′- 3′)	Size (bp)
Event-specific PCR for ZDRF8	ZDRF8-LB-F1	CGTCCGCAATGTGTTATTAAGTTGTCTA	290
	ZDRF8-LB-R1	AGTGCCAATACATACGCAACTGTTGCAG	
Event-specific PCR for nCX-1	nCX-1-RB-F1	AGCTGGCGTAATAGCGAAGAGG	362
	nCX-1-RB-R1	CAACTGCGTCGTCTGGAGATTCAACTG	

and a 16-hour day/8-hour night photoperiod. Larval survival was monitored daily at a fixed time, and the number of live corn borers was recorded. The experiment concluded when the survival rate of larvae in the transgenic maize ZDRF8×nCX-1 group reached 0%.

Field Evaluation of Stacked Transgenic Maize

The stacked maize ZDRF8×nCX-1 and its non-transgenic counterpart were cultivated in Deqing, Zhejiang Province, following a randomized block design with three replications. Each block was 4 m long and consisted of 8 corn rows with 6 rows of ZDRF8×nCX-1 maize and 2 rows of non-GM maize. Based on previous tests showing no significant difference in the yields of non-transgenic maize and ZDRF8×nCX-1 under normal field management, only two rows of non-transgenic maize were designated as the control for trait detection in this design. Throughout the growing period, no insecticides were applied. At the 3–4 leaf stage of maize, ZDRF8×nCX-1, non-GM maize, and weed species present in the local production system were sprayed with herbicide. Herbicide treatments included 675 g ae ha^{-1} glyphosate +4.5 g ai ha^{-1} flazasulfuron, 1350 g ae ha^{-1} glyphosate +9 g ai ha^{-1} flazasulfuron, 675 g ae ha^{-1} glyphosate +420 g ai ha^{-1} MCPA, and 1350 g ae ha^{-1} glyphosate +840 g ai ha^{-1} MCPA. A control group with no herbicide application was also included. At the maturity stage, 4 rows of ears from the middle of the ZDRF8×nCX-1 maize plot were harvested to analyze the yield. The harvest area was 8 m^2 per block. The weight of harvested corn grains and moisture level were measured and then converted into a standard moisture level (15%) to calculate the average yield per mu.

Total RNA Extraction and Transcriptome Sequencing Library Construction

Five plants at the same developmental stage (seven to eight leaves) were selected randomly from each variety for the extraction of total RNA. Total RNA from leaves was extracted using the RNAprep Pure Plant Plus Kit (Cat.No. DP441, TIANGEN BIOTECH, Beijing). The concentration of total RNA was determined using a Qubit 2.0 Fluorometer. The integrity of total RNA was assessed by agarose gel electrophoresis. All RNA samples extracted from the four maize varieties were deemed suitable. A mixture of RNA samples from five plants of each maize variety was used to sequence the transcriptome.

The transcriptome sequence library was prepared using the NEBNext® UltraTM RNA Library Prep Kit for Illumina (NEB, America) following the manufacturer's instructions. Briefly, 100 ng of purified mRNA was fragmented using Random Primers and First Strand Synthesis Reaction Buffer (5×). The short mRNA fragments were used as templates to synthesis first-strand cDNAs using Murine RNase Inhibitor and Protoscript II Reverse Transcriptase, then second-strand cDNA was synthesized using Strand Synthesis Reaction Buffer (10×) and Second Strand Synthesis Enzyme Mix. The double-strand cDNA was purified using AMPure XP Beads (Agencourt, America), followed by end repair, single nucleotide (A) addition, adaptors ligation and U excision with User Enzyme Mix. Target cDNA fragments of 300–500 bp were recovered by agarose gel electrophoresis and enriched by PCR amplification to generate a library for transcriptome sequencing using the Illumina Nova platform.

Analysis of Sequencing Data

The raw fluorescent image documents obtained from the Illumina Nova platform were converted into sequenced reads using CASAVA Base Calling software. The Raw data underwent quality control using Trimmomatic v0.32 software to remove adapter sequences, reads containing N bases, low-quality reads (Q score < 20), and reads shorter than 50nt in length, along with their paired reads. Clean reads were then obtained and aligned to the B73 reference transcriptome using Bowtie 2 v2.1.0 software with the default parameters.

Gene expression levels were calculated in Reads Per Kilobase of exon model per Million mapped reads (RPKM) based on the number of reads mapped to the reference sequence. The MA-plot-based method with the random sampling model (MARS) in the DEGseq v1.20.0 software package was used to evaluate the expression levels of each transcript. The screening conditions for differentially expressed genes were set at |Fold change|>2,

FDR (q value) <0.001. The identified differentially expressed genes were mapped to Gene Ontology (GO) terms to perform gene ontology annotation and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to identify associated biochemical pathways.

RT-qPCR (Real Time Quantitative PCR)

Real-time quantitative PCR (RT-qPCR) was used to analyze the differentially expressed genes (DEGs) in different backgrounds of maize lines. Total RNA was extracted using the Easy Plant RNA Kit (DR040650, YSD-Bio, Hangzhou, China). Approximately 1 μg of the extracted RNA was used as a template to synthesize cDNA with HiScript II Q RT SuperMix for qPCR Kit with gDNA wiper (R223, Vazyme, Nanjing, China). The sequences of the primers used for RT-qPCR are listed in Supplementary Table S1. RT-qPCR was performed using the AceQ qPCR SYBR Green Master Mix Kit (Q111–02, Vazyme, Nanjing, China). The RT-qPCR protocol consisted of the following steps: 5 min at 95°C, followed by 39 cycles of 95°C for 10 sec and 60°C for 30 sec. The *zSSIIb* gene was used as an internal control. RT-qPCR data was calculated according to the $2^{-\Delta\Delta CT}$ method.[22] Three biological replicates and three technical replicates were performed.

Nutritional Composition Analysis

The proximates, fatty acids, amino acids, vitamins and minerals were analyzed at the Hangzhou Center of Inspection and Testing for Quality and Safety of Agricultural and Processed Products, Ministry of Agriculture and Rural Affairs, P.R. China. Triplicate samples for each type of maize were selected for compositional analysis.

The contents of moisture, ash, protein, crude fat and starch were determined according to Chinese standards GB 5009.3–2016, GB 5009.4–2016, GB 5009.5–2016, GB 5009.6–2016, and GB 5009.9–2016, respectively.

Crude fiber was analyzed following Chinese standard GB/T 5009.10–2003.

Individual fatty acids were quantified using gas-liquid chromatography in accordance with Chinese standard GB 5009.168–2016.

Amino acids were analyzed using an automatic amino acid analyzer after protein hydrolysis with HCl, as per Chinese standard GB 5009.124–2016.

Thiamin (vitamin B1) and riboflavin (vitamin B2) contents were determined by fluorometric methods following the Chinese standard GB 5009.84–2016 and GB 5009.85–2016, respectively. Vitamin E was quantified by high-performance liquid chromatography according to GB 5009.82–2016. The total vitamin E content was calculated as the sum of α-, γ-, and δ-tocopherols.

The levels of phosphorus, zinc, copper, iron, magnesium, manganese, potassium, and calcium were measured using inductively coupled plasma optical emission spectrometry (ICP-OES) in compliance with GB 5009.268–2016.

Data analysis was performed using GraphPad Prism 9.0.0 software. The nutritional composition values of ZDRF8×nCX-1 and the non-transgenic control maize (RF1) were expressed as mean ± standard deviation. Statistical significance ($p < 0.05$) was determined by comparing the mean values of ZDRF8×nCX-1 and the control maize. To minimize false positives, the false discovery rate (FDR) methodology was applied. When the FDR-adjusted p-value exceeded 0.05 despite a raw p-value indicating a significance, the difference was considered likely to be a false positive. Additionally, the ZDRF8×nCX-1 maize values were compared to the literature range, revealing a statistically significant difference (adjusted $p < 0.05$). These literature ranges were derived from publicly available crop composition data, including the transgenic parents ZDRF8 and nCX-1.

Results

Generation and Molecular Characterization of Stacked Transgenic Maize ZDRF8×nCX-1

In this study, the stacked transgenic maize ZDRF8×nCX-1 was obtained by crossing insect-resistant maize ZDRF8 and herbicide-tolerant maize nCX-1.

Event-specific PCR detection, which targets the flanking junction sequence between the exogenous

DNA and the plant genome, enables rapid and accurate identification of different transgenic crop lines. The event-specific PCR results demonstrated that the stacked transgenic maize line ZDRF8×nCX-1 and its parental lines, ZDRF8 and nCX-1, exhibited identical target DNA bands (Figure 1).

Reverse transcription PCR (RT-PCR) amplified the *cry1Ab*, *cry2Ab* and *g10evo-epsps* genes from the parent ZDRF8, and the *cp4 epsps* and *P450-N-Z1* genes from the parent nCX-1. All target DNA bands corresponding to the *cry1Ab*, *cry2Ab*, *g10evo-epsps*, *cp4 epsps* and *P450-N-Z1* genes were amplified from the stacked transgenic maize ZDRF8×nCX-1 (Figure 2).

ELISA data revealed that the Cry1Ab content was 12.52 μg/g and 15.37 μg/g in the leaves of transgenic maize ZDRF8 and ZDRF8×nCX-1, respectively. The Cry2Ab content was 0.85 μg/g and 1.02 μg/g in the leaves of transgenic maize ZDRF8 and ZDRF8×nCX-1, respectively. The G10evo-EPSPS content was 1.94 μg/g and 0.99 μg/g in the leaves of transgenic maize ZDRF8 and ZDRF8×nCX-1, respectively. The CP4 EPSPS expression levels in the leaves of transgenic maize ZDRF8 and ZDRF8×nCX-1 were 71.85 μg/g and 77.23 μg/g, respectively. The P450-N-Z1 expression levels in the leaves of transgenic maize nCX-1 and ZDRF8×nCX-1 were 0.46 μg/g and 0.50 μg/g, respectively (Figure 3).

The results above indicated that the insertion sites of foreign genes in the maize genome remained unchanged during the breeding process. Furthermore, the foreign genes were successfully expressed at both the RNA and protein levels in the stacked transgenic maize ZDRF8×nCX-1.

Trait Analysis of Stacked Transgenic Maize ZDRF8×nCX-1

The *g10evo-epsps* and *cp4 epsps* genes, derived from its parental lines ZDRF8 and nCX-1, are present in transgenic maize ZDRF8×nCX-1, conferring glyphosate tolerance. The *P450-N-Z1* gene, originating from nCX-1, provides ZDRF8×nCX-1 with flazasulfuron tolerance. Glyphosate spray experiment revealed that non-transgenic maize RF1 died 10 days after treatment, while ZDRF8×nCX-1 and its parental lines ZDRF8 and nCX-1, exhibited glyphosate tolerance (Figure 4a). Similarly, flazasulfuron spray experiment demonstrated that ZDRF8×nCX-1 and its parental line nCX-1 were tolerant to flazasulfuron, whereas ZDRF8 and non-transgenic maize RF1 died 10 days after treatment (Figure 4b).

Laboratory bioassay results indicated that all corn borers died within 3 days of feeding on young leaves from transgenic maize ZDRF8×nCX-1 and ZDRF8. In contrast, corn borers fed on young leaves from nCX-1 and non-transgenic maize RF1 exhibited a survival rate exceeding 90% (Figure 4d).

These results demonstrate that ZDRF8×nCX-1 possesses both herbicide tolerance and pest resistance traits.

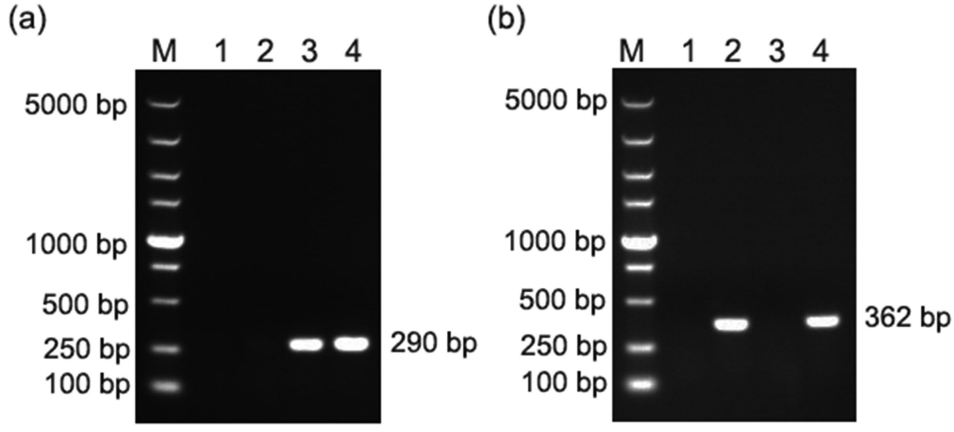

Figure 1. Event-specific PCR detection of transgenic maize ZDRF8, nCX-1, ZDRF8×nCX-1 and non-transgenic maize RF1. M, DL5000 DNA marker. (a) Event-specific PCR detection using primers ZDRF8-LB-F1 and ZDRF8-LB-R1. Lanes 1–4 represent the amplification of RF1, nCX-1, ZDRF8 and ZDRF8×nCX-1. (b) Event-specific PCR detection using primers nCX-1-RB-F1 and nCX-1-RB-R1. Lanes 1–4 represent the amplification of RF1, nCX-1, ZDRF8 and ZDRF8×nCX-1.

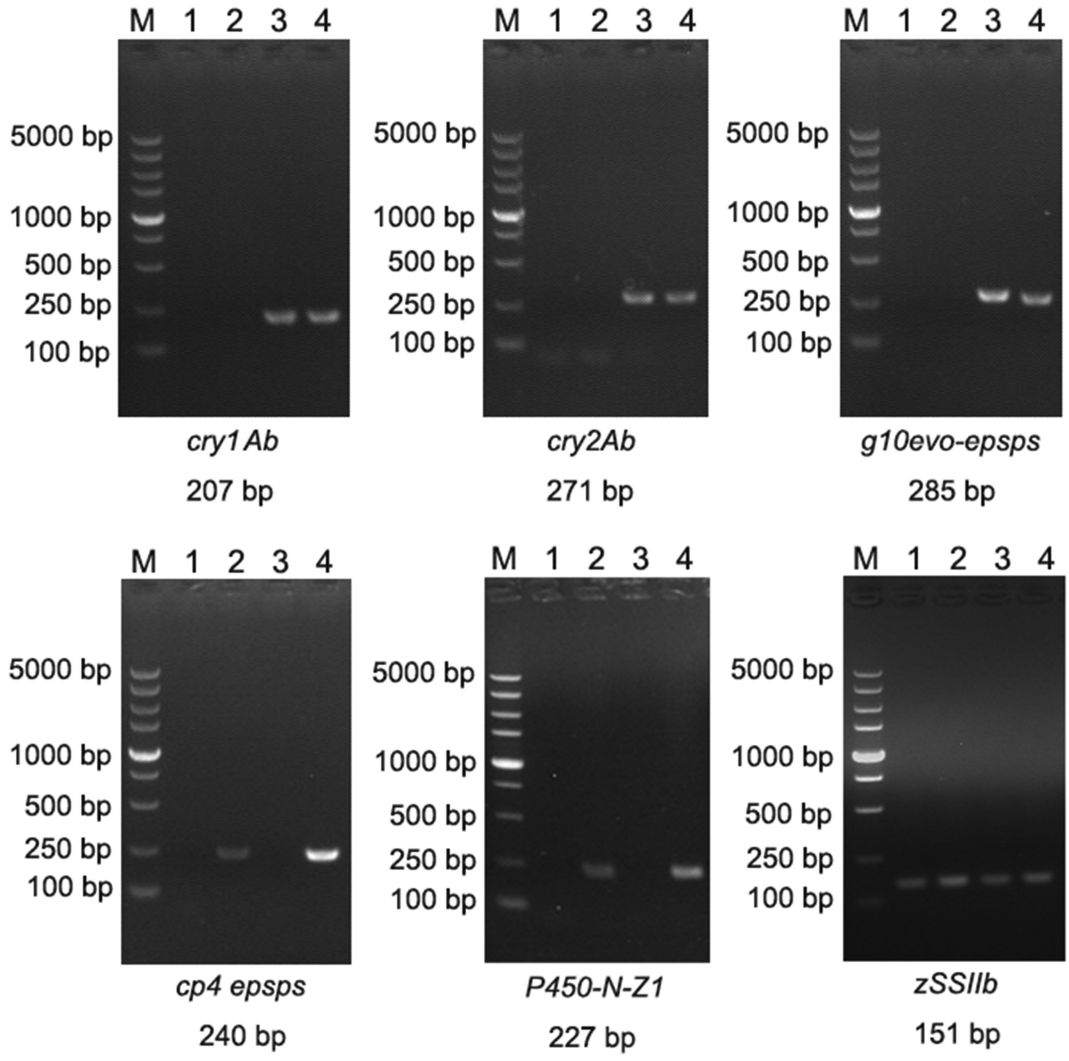

Figure 2. Reverse transcription-polymerase chain reaction (RT-PCR) detection of the exogenous genes *cry1Ab*, *cry2Ab*, *g10evo-epsps*, *cp4 epsps* and *P450-N-Z1*. The *zSSIIb* gene was used as a reference gene. M, DL5000 DNA marker. Lanes 1–4 represent the amplification of RF1, nCX-1, ZDRF8 and ZDRF8×nCX-1.

Field Evaluation of Stacked Transgenic Maize ZDRF8×nCX-1

Field evaluation of ZDRF8×nCX-1 was conducted in Deqing (30°34′32″ N latitude, 119°55′53″ E longitude). No pest management measures were applied throughout the growing period. At the 3–4 leaf stage, ZDRF8×nCX-1, non-transgenic maize, and the weeds present in the local production system were sprayed with a mixture of glyphosate (G) and flazasulfuron (F) or glyphosate (G) and MCPA (M). The doses of the G + F and G + M were 675 g ae ha^{-1} G +4.5 g ai ha^{-1} F, 1350 g ae ha^{-1} G +9 g ai ha^{-1} F, 675 g ae ha^{-1} G +420 g ai ha^{-1} M, 1350 g ae ha^{-1} G +840 g ai ha^{-1} M, respectively. One week after herbicide application, the leaves of non-transgenic maize and weeds exhibited wilting, necrosis, and clear symptoms of damage compared to the no herbicide treatment control (Figure 5a-f). After spraying 675 g ae ha^{-1} G +4.5 g ai ha^{-1} F and 1350 g ae ha^{-1} G +9 g ai ha^{-1} F, ZDRF8×nCX-1 maize grew normally (Figure 5a,b). Similarly, no crop injury was observed in the ZDRF8×nCX-1 maize following of 675 g ae ha^{-1} G +420 g ai ha^{-1} M and 1350 g ae ha^{-1} G +840 g ai ha^{-1} M (Figure 5c,d). Meanwhile, non-transgenic maize plants exhibited damage caused by lepidopteran pests under natural infestation (Figure 5e-h). Importantly, there were no significant differences in the yield of ZDRF8×nCX-1 under different herbicide treatments (Figure 5i). In contrast, the yield of ZDRF8×nCX-1 decreased due to the weed infestation in the no herbicide treatment (Figure 5i).

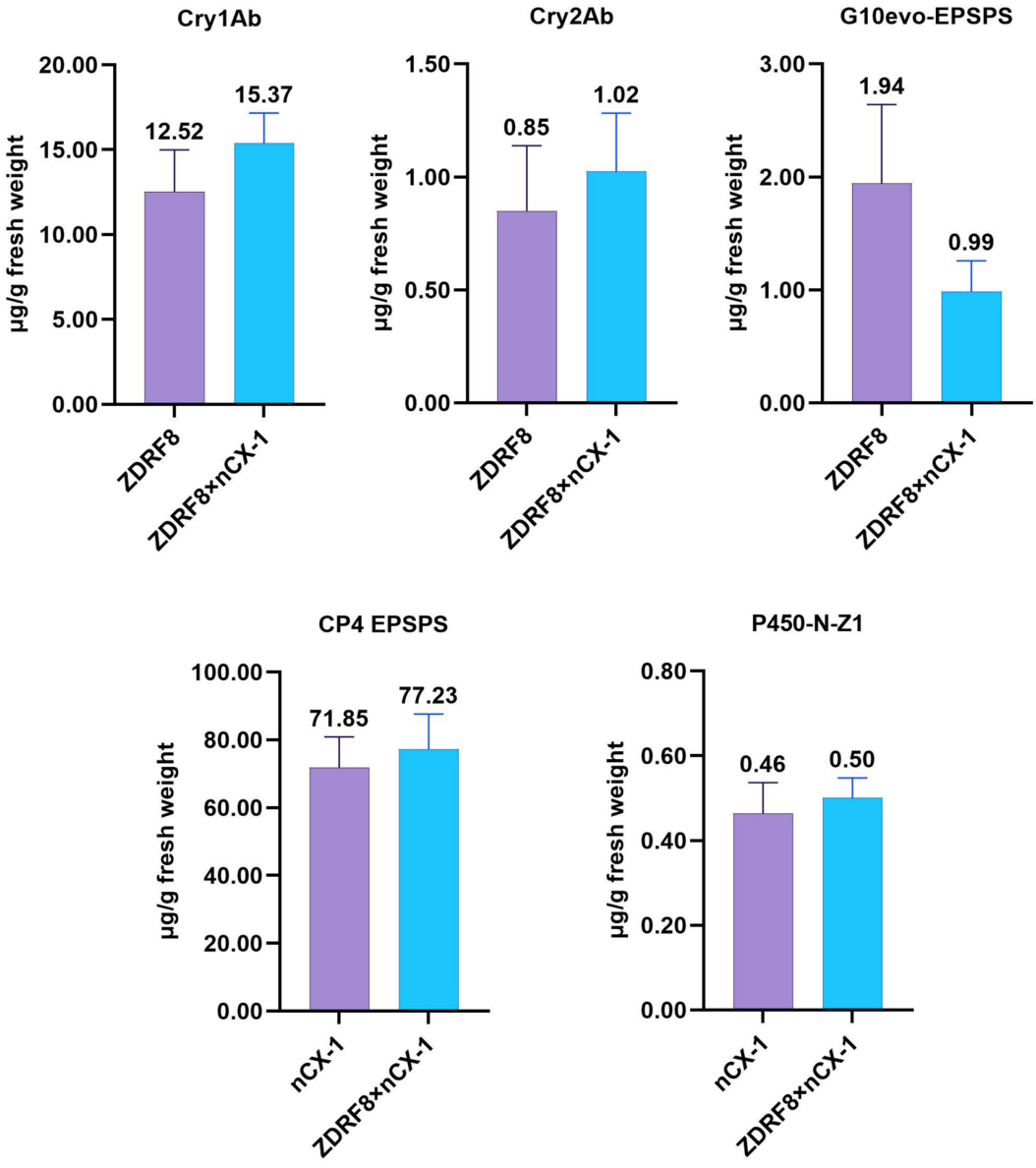

Figure 3. Protein content of exogenous proteins detected by ELISA in the leaves of different transgenic maize lines.

Transcriptomic Analysis

RNA-Sequencing and Sample Transcriptome Mapping

After raw data quality filtering, approximately 20–21 million clean reads were obtained from four maize samples. Using the B73 maize transcriptome as a reference, approximately 56%–71% of the clean reads were successfully mapped to the reference genome. Among the mapped reads, uniquely mapped reads constituted 58–60%, while multiple mapped reads accounted for 39–41%. These results indicate that the transcriptome mapping information obtained from the maize samples is sufficient for further analysis, as detailed in Table 3.

Differentially Expressed Genes Analysis

The number of differentially expressed genes (DEGs) in different comparison groups is summarized in Table 4. There were 467 DEGs identified in the comparison group RF1/ZDRF8, including 270 up-regulated genes and 197 down-regulated genes (Supplementary Figure S2a). In the comparison group RF1/nCX-1, 685 up-regulated genes and 400 down-regulated genes were identified (Supplementary Figure S2b). In the comparison group RF1/ZDRF8×nCX-1, 615 DEGs were identified, including 261 up-regulated and 354 down-regulated genes (Supplementary Figure S2c). There were 558 DEGs identified in the comparison

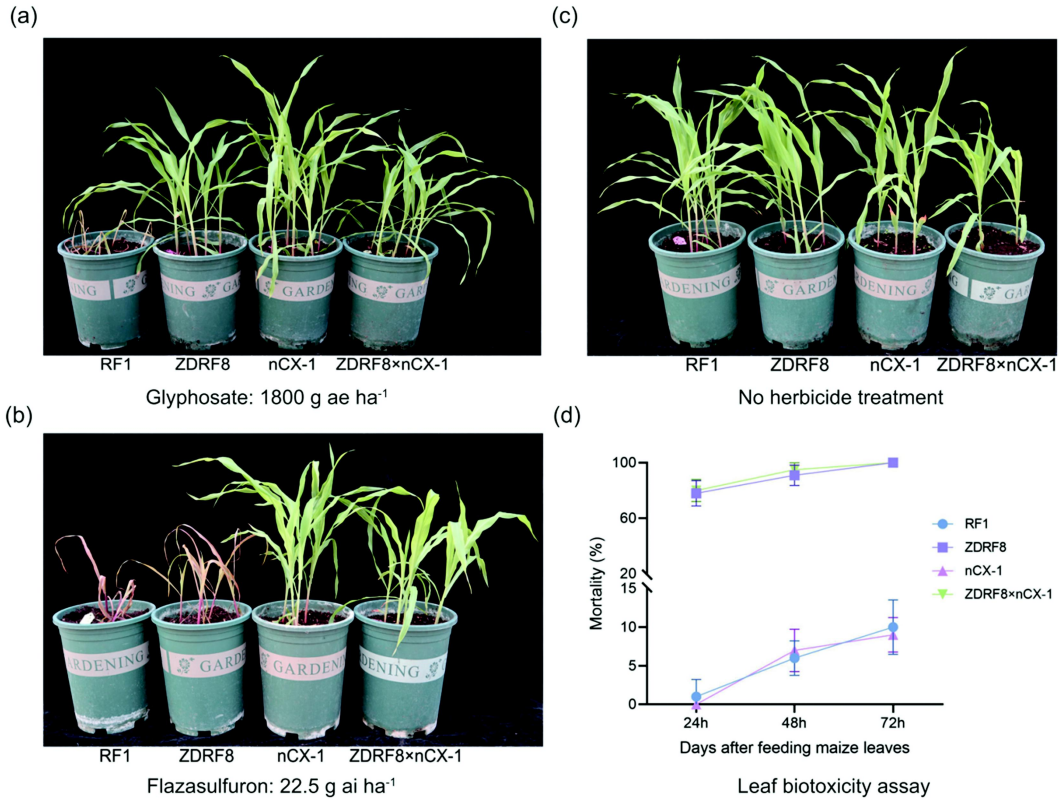

Figure 4. Herbicide tolerance and insect resistance analysis of transgenic maize ZDRF8, nCX-1, ZDRF8×nCX-1 and non-transgenic maize RF1. (a) The four maize lines were treated with glyphosate at a rate of 1800 g ae ha^{-1}. (b) The four maize lines were treated with flazasulfuron at a rate of 22.5 g ai ha^{-1}. (c) The four maize lines without herbicide treatment. (d) Mortality of corn borer larvae after feeding on different maize lines.

group ZDRF8×nCX-1/ZDRF8, 365 genes were up-regulated and 193 gens were down-regulated (Supplementary Figure S2d). In the comparison group ZDRF8×nCX-1/nCX-1, 592 up-regulated genes and 290 down-regulated genes were identified (Supplementary Figure S2e). A comparison of nCX-1 with ZDRF8 identified 499 DEGs, of which 210 were up-regulated and 289 were down-regulated (Supplementary Figure S2f).

Among RF1/ZDRF8, RF1/nCX-1 and RF1/ZDRF8×nCX-1 pairwise comparisons, 182 DEGs were simultaneously identified (Figure 6a). Among the pairwise comparisons of ZDRF8×nCX-1 with its parental lines ZDRF8 and nCX-1, 13 common DEGs were identified across all three groups (Figure 6b). Transgenic processes had a greater effect on gene expression (182 common DEGs), indicating that the gene expression pattern of transgenic materials was significantly different from that of non-transgenic material. The effect of hybrid stacking on gene expression was relatively small (13 common DEGs), indicating that the gene expression pattern of hybrid offspring was more similar to that of parents. These results revealed that transgenes had a greater effect on gene expression than hybrid stacking.

The key metabolic pathways in maize include photosynthesis, sugar metabolism, nitrogen metabolism, lipid metabolism and secondary metabolism. Phenylalanine, an aromatic amino acid, plays a pivotal role in maize growth, development, and stress adaptation. It serves as a metabolic hub connecting primary and secondary metabolism, enabling maize to synthesize essential compounds for structural integrity, defense, and environmental interactions. Photosynthesis is the cornerstone of energy production and growth in maize, enabling it to thrive as one of the world's most productive crops. Valine, leucine, and isoleucine are branched-chain amino acids, and their biosynthesis in maize is crucial for multiple aspects of the plant's growth, development, and survival. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that the DEGs between

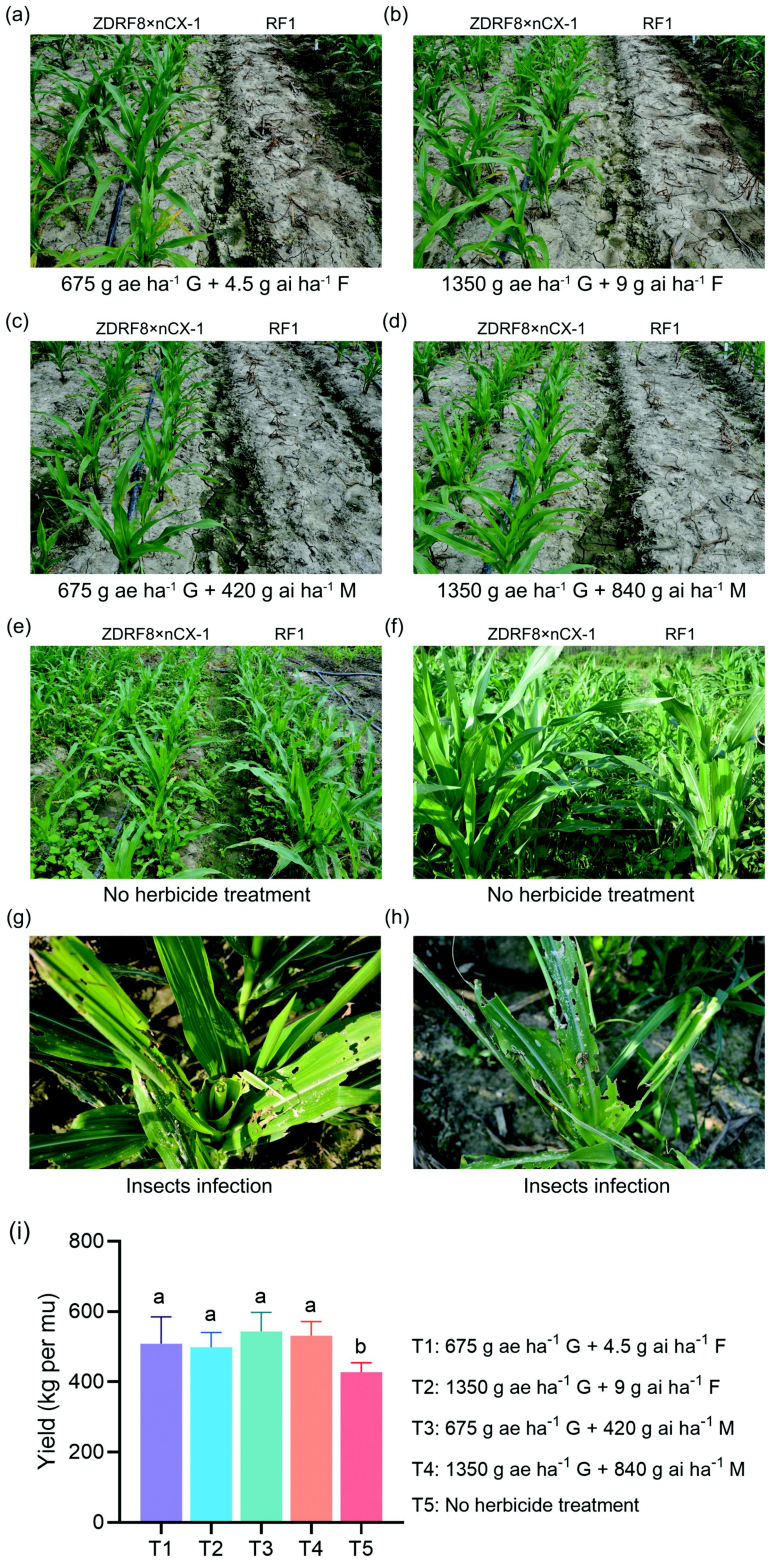

Figure 5. Field evaluations of ZDRF8×nCX-1 and the non-transgenic maize RF1. (a) Phenotypes of ZDRF8×nCX-1, control plants, and weeds 7 days after application of 675 g ae ha^{-1} glyphosate + 4.5 g ai ha^{-1} flazasulfuron. (b) Phenotypes of ZDRF8×nCX-1, control plants, and weeds 7 days after application of 1350 g ae ha^{-1} glyphosate + 9 g ai ha^{-1} flazasulfuron. (c) Phenotypes of ZDRF8×nCX-1, control plants, and weeds 7 days after application of 675 g ae ha^{-1} glyphosate + 420 g ai ha^{-1} MCPA. (d) Phenotypes of ZDRF8×nCX-1, control plants, and weeds 7 days after application of 1350 g ae ha^{-1} glyphosate + 840 g ai ha^{-1} MCPA. (e) Phenotypes of ZDRF8×nCX-1, control plants, and weeds 23 days after sowing without herbicide application. (f) Phenotypes of ZDRF8×nCX-1, control plants, and weeds 38 days after sowing without herbicide application. (g, h) Field imaging of non-transgenic maize exposed naturally to insects. (i) Yield of ZDRF8×nCX-1 under different herbicide treatments.

Table 3. RNA-seq and maize samples transcriptome mapping results.

Materials	ZDRF8	nCX-1	ZDRF8×nCX-1	RF1
Raw reads	22044561	22500184	22447833	22011600
Clean reads	20227735	20320893	20518642	20631526
Mapped reads	1198332 (59%)	1143813 (56%)	1161946 (57%)	14578707 (71%)
Unique mapped reads	7139220 (59%)	6770673 (59%)	7011897 (60%)	8472718 (58%)
Multiple mapped reads	4844104 (40%)	4667463 (40%)	4607570 (39%)	6105989 (41%)

Table 4. Number of identified DEGs in different comparison groups.

Comparison group	Up-regulated	Down-regulated	Total number of DEGs
RF1/ZDRF8	270(57.82%)	197(42.18%)	467
RF1/nCX-1	685(63.13%)	400(36.87%)	1085
RF1/ZDRF8×nCX-1	261(42.44%)	354(57.56%)	615
ZDRF8×nCX-1/ZDRF8	365(65.41%)	193(34.59%)	558
ZDRF8×nCX-1/nCX-1	592(67.12%)	290(32.88%)	882
nCX-1/ZDRF8	210 (42.08%)	289(57.92%)	499

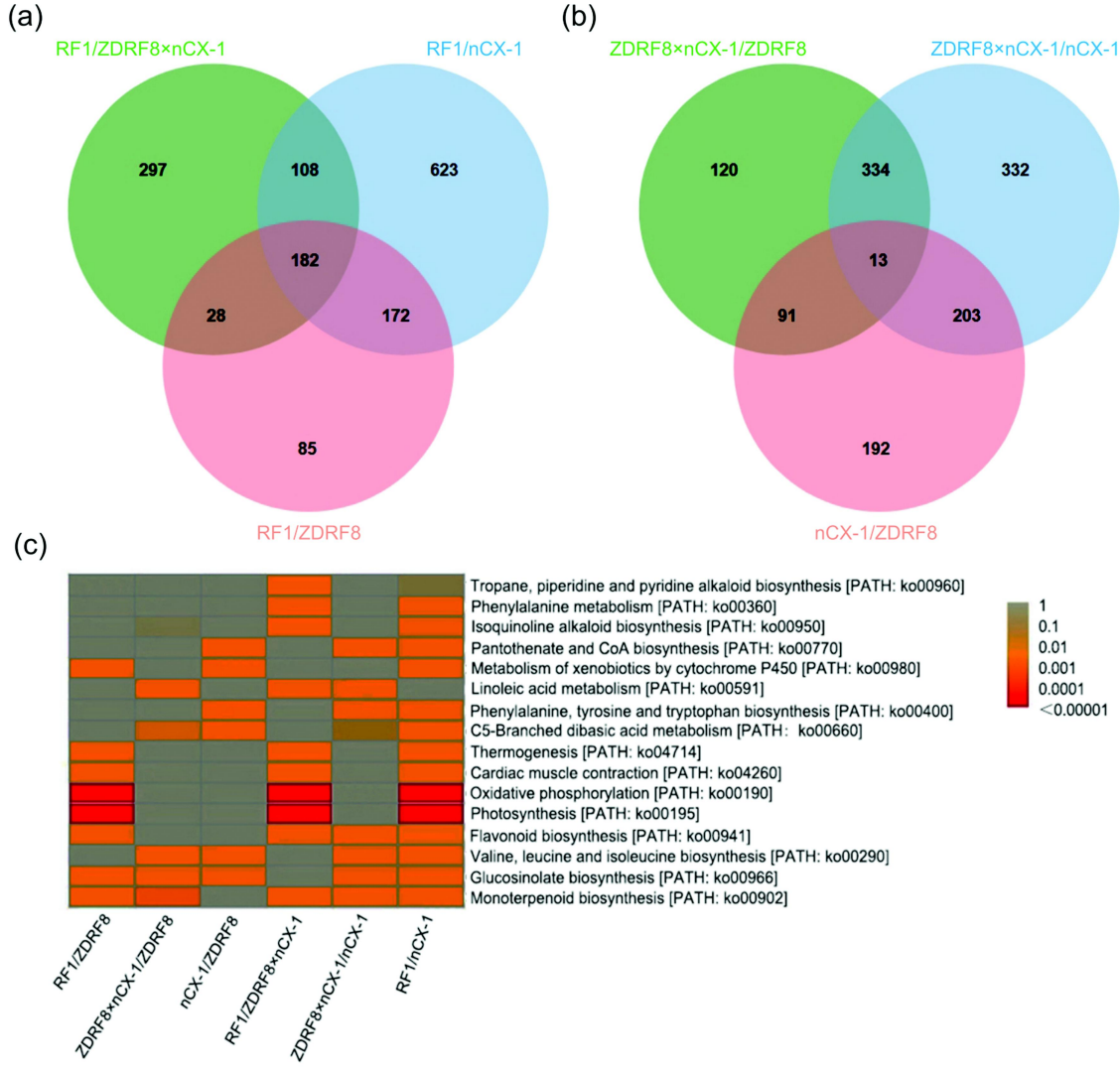

Figure 6. Differential gene expression transcriptome analysis. (a) Venn diagram of differentially expressed genes across pairwise comparisons among 3 groups of GM/non-gm maize. (b) Venn diagram of differentially expressed genes across pairwise comparisons among three transgenic maize lines resulting from stack breeding. (c) KEGG pathway enrichment analysis of differentially expressed genes between four maize lines.

ZDRF8×nCX-1 and its parental lines ZDRF8 and nCX-1 exhibited the same enrichment trends in the phenylalanine metabolism pathway, photosynthesis, as well as the valine, leucine and isoleucine biosynthesis pathways (Figure 6c). This finding suggests that in these key metabolic and physiological processes, the stacked maize ZDRF8×nCX-1 inherits and maintains a similar functional orientation to its parental lines.

Analysis of Genes Related to Shikimate Pathway

The gene-stacked maize ZDRF8×nCX-1 incorporates insect resistance and herbicide tolerance traits through hybrid breeding. Insect resistance is conferred by the *cry1Ab* and *cry2Ab* genes derived from ZDRF8. These Bt genes, which are not naturally present in plants, do not have any known metabolic functions. Glyphosate tolerance is achieved through the introduction of the *g10evo-epsps* and *cp4 epsps* genes, which encode the enzyme 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS) from ZDRF8 and nCX-1, respectively. EPSPS plays a critical role in the sixth step of the shikimate pathway, a seven-step metabolic process responsible for synthesizing aromatic amino acids (tryptophan, phenylalanine, and tyrosine) in plants.[23] Other enzymes involved in this pathway include 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), 3-dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase (DHQD), shikimate dehydrogenase (SDH), shikimate kinase (SK), and chorismate synthase (CS).[24] The *aroF*, *aroB*, *aroD*, and *aroE* genes encode DAHPS, DHQS, DHQD, and SDH, respectively. The *aroK* gene encodes shikimate kinase, the fifth enzyme in the shikimate pathway, upstream of EPSPS. The *aroA* gene, encoding EPSPS, is involved in the penultimate step of the shikimate pathway. The *aroC* gene encodes chorismate synthase, which catalyzes the EPSP to chorismate. The transcriptome levels of genes encoding shikimate pathway enzymes in different maize lines were analyzed (Table 5). For the *aroF* gene, no significant differential expression was observed between RF1/ZDRF8, RF1/nCX-1, RF1/ZDRF8×nCX-1, ZDRF8×nCX-1/ZDRF8; however, it was up-regulated in ZDRF8×nCX-1 and ZDRF8 compared to nCX-1. No significant differences were observed in the expression of the *aroB*, *aroD*, *aroE*, *aroK*, *aroA*, and *aroC* genes among transgenic maize ZDRF8, nCX-1, ZDRF8×nCX-1, and non-transgenic maize RF1. These findings suggest that the integration of genes encoding the EPSPS protein is unlikely to significantly impact the shikimate pathway or the production of aromatic amino acids in plants in the absence of glyphosate selection pressure.

Table 5. Gene expression analysis of shikimate pathway enzymes between different maize lines.

Comparison group	Gene	Log_2 fold change	p-value	Differential expression
RF1/ZDRF8	*aroF*	−0.327	2.01E−06	NO
	aroB	0.189	0.015	NO
	aroD	1.013	3.62E−05	NO
	aroE	0.449	0.0003	NO
	aroK	−0.590	1.42E−72	NO
	aroA	0.065	0.492	NO
	aroC	0.223	6.44E−05	NO
RF1/nCX-1	*aroF*	0.771	5.61E−20	NO
	aroB	0.766	4.37E−62	NO
	aroD	1.761	4.25E−10	NO
	aroE	0.679	1.90E−07	NO
	aroK	0.124	0.122	NO
	aroA	0.446	1.10E−05	NO
	aroC	0.661	1.64E−27	NO
RF1/ZDRF8×nCX-1	*aroF*	0.906	3.07E−47	NO
	aroB	0.110	0.156	NO
	aroD	0.383	0.083	NO
	aroE	0.051	0.663	NO
	aroK	0.471	3.14E−08	NO
	aroA	−0.289	0.001	NO
	aroC	0.117	0.035	NO
ZDRF8×nCX-1/ ZDRF8	*aroF*	0.579	2.03E−20	NO
	aroB	0.079	0.349	NO
	aroD	0.629	0.024	NO
	aroE	0.398	0.003	NO
	aroK	−0.525	1.26E−52	NO
	aroA	0.353	0.0001	NO
	aroC	0.106	0.008	NO
ZDRF8×nCX-1/ nCX-1	*aroF*	1.677	1.35E−106	Up-regulated
	aroB	0.910	2.95E−20	NO
	aroD	1.378	1.68E−05	NO
	aroE	0.629	6.89E−06	NO
	aroK	−0.347	0.0002	NO
	aroA	0.735	7.92E−13	NO
	aroC	0.544	1.47E−16	NO
nCX-1/ZDRF8	*aroF*	−1.098	3.53E−39	Down-regulated
	aroB	−0.831	5.77E−17	NO
	aroD	−0.748	0.035	NO
	aroE	−0.231	0.120	NO
	aroK	−0.094	0.271	NO
	aroA	−0.382	0.0004	NO
	aroC	−0.438	4.88E−11	NO

Differential Expression Analysis of Cytochrome P450 Family Genes

Cytochrome P450 monooxygenases (CYP450s) constitute the largest superfamily of enzyme proteins. These enzymes are ubiquitous in the genomes of virtually all organisms, particularly in plants, where they are present in significant numbers. CYP450 enzymes, apart from their pivotal role in herbicide metabolism and detoxification, are the largest enzyme

family responsible for synthesizing lignin, pigments, fatty acids, hormones, and signaling molecules.[25,26] For example, oxidized fatty acids have significant biological functions, and CYP450s play a major role in the production of hydroxy-fatty acids, such as those in the CYP703, CYP704, CYP81, CYP86, and CYP96 families.[27–30] CYP450s are also crucial in the synthesis of several secondary compounds, such as the hydroxylation of the aromatic cinnamates.[29] The gene *P450-N-Z1*, also known as *CYP81A69*, has been isolated from Bermuda grass and shown to confer tolerance to flazasulfuron by facilitating its degradation.[31]

After a thorough examination, we identified significant differences in the expression levels of P450 family genes across various genetic backgrounds in maize. Specifically, we compared the transcription levels of these genes in RF1/nCX-1, RF1/ZDRF8, RF1/ZDRF8×nCX-1, ZDRF8×nCX-1/nCX-1, ZDRF8×nCX-1/ZDRF8, and nCX-1/ZDRF8. Our results revealed 13 genes that demonstrated differential expression patterns between stacked, single GM events, as well as their non-transgenic isogenic counterparts (Table S2). The functions of these genes primarily involve heme binding, iron ion binding, monooxygenase activity, and oxidoreductase activity, specifically acting on paired donors with either incorporation or reduction of molecular oxygen. Six P450 genes, *Zm00001d015589*, *Zm00001d038555*, *Zm00001d047495*, *Zm00001d020628*, *Zm00001d039310*, *Zm00001d012304*, which were differentially expressed in ZDRF8×nCX-1 and its parents were selected for RT-qPCR analysis to detect their transcription levels in other genetic backgrounds, PH4CV, ZDRF8-PH4CV, nCX-1-PH4CV, and ZDRF8×nCX-1-PH4CV. We found that the relative expression levels of the six differentially expressed genes with the PH4CV background were slightly different from those of the transcriptome with the RF1 background, indicating that, in addition to genetic modification, different genetic backgrounds also affect transcript profiling (Figure 7). These findings provide crucial insights into the genetic modifications and background that impact the expression of P450 family genes in maize. Understanding these patterns is crucial for assessing the potential consequences of GM modifications on the overall biology and agronomic performance of the crop.

Nutritional Compositions Analysis in ZDRF8×nCX-1 Grain

Due to the natural variation in composition values across different maize lines, the composition values of ZDRF8×nCX-1 were compared not only with those of the transgenic parents and non-transgenic control maize RF1 but also with published values in OECD (2002), the ILSI Crop Composition Database (2023) and other periodicals to determine whether the observed differences exceeded the range of natural variation.[32,33] The composition values of the transgenic parents ZDRF8 and nCX-1 were shown to be substantially equivalent to their non-GM counterpart, respectively.[34,35] This study present only the composition values of ZDRF8×nCX-1 and RF1 (Figure 8). The values of moisture, crude fiber, palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), eicosenoic acid (C20:1), aspartic acid, threonine, glutamic acid, proline, leucine, histidine, vitamin E, vitamin B2, copper, magnesium, and potassium in ZDRF8×nCX-1 differed significantly from those of the non-transgenic control maize RF1. However, all these values fell within the reference range of natural variation. Therefore, the observed differences between ZDRF8×nCX-1 and RF1 unlikely to be biologically relevant. For detailed information, refer to Supplementary Table S3–S7.

Discussion

Stacked trait GM crops, incorporating different Bt genes and herbicide tolerance genes, provide farmers with multiple solutions to combat pests and weeds. The stacked GM maize ZDRF8×nCX-1 showed high tolerance to glyphosate, flazasulfuron, and MCPA, as well as resistance to lepidopteran pests under natural infection. Therefore, we anticipate that the commercial cultivation of ZDRF8×nCX-1 maize will enhance pest management and weed control in the maize production system. However, for the safe use of stacked GM maize ZDRF8×nCX-1, the potential unintended effects of combining transgene by conventional breeding must be carefully investigated.

In traditional breeding, gene interaction is a common and significant phenomenon.[36] A metabolic or physiochemical interaction resulting from the combination of the transgene products is the sole type of interaction observed in stack

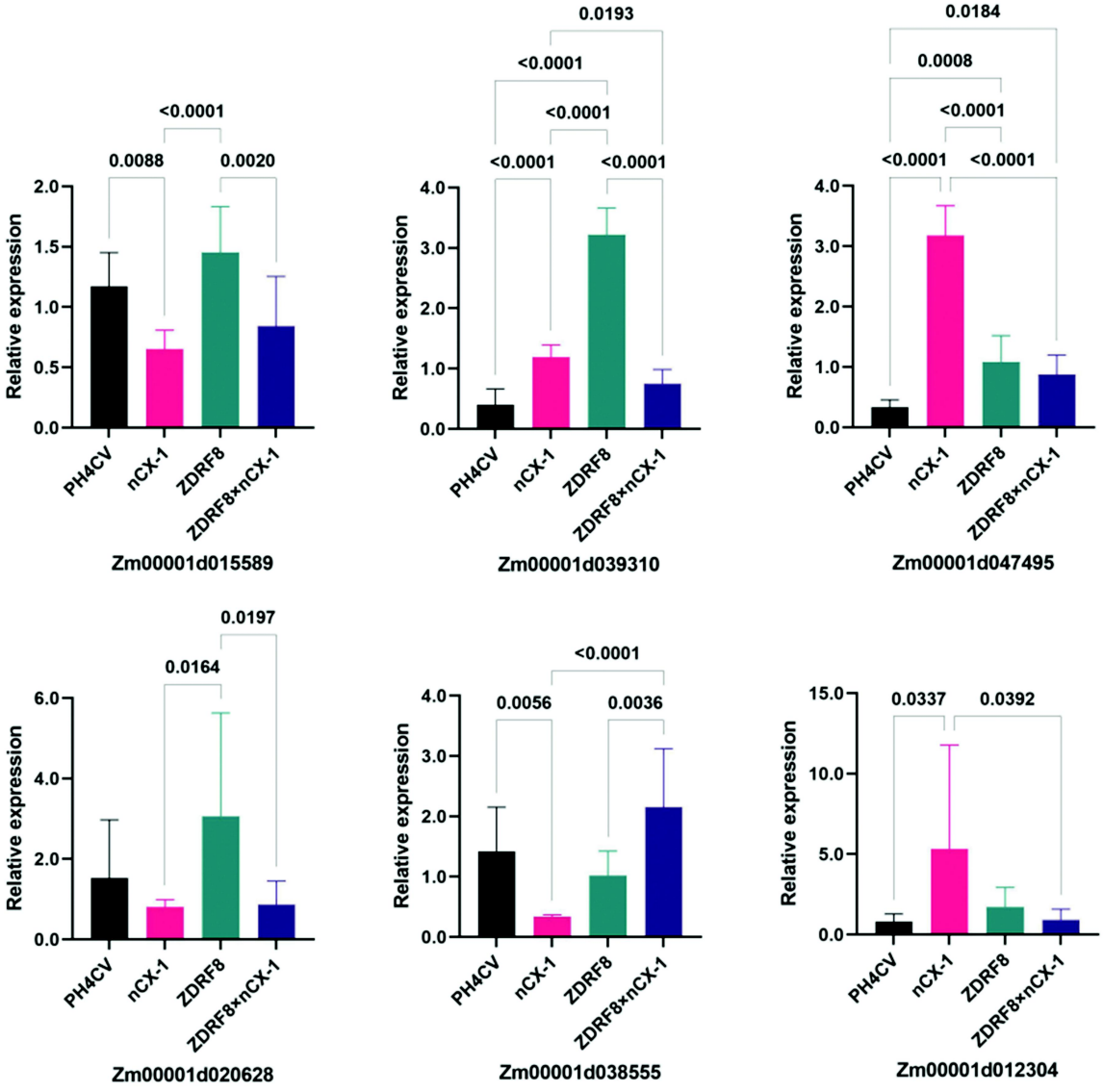

Figure 7. RT-qPCR analysis of the relative gene expression of differentially expressed genes among different maize lines with the PH4CV background. The maize *zSSIIb* gene was used as an internal control to normalize expression levels.

breeding of transgenic plants that differs from traditional breeding. The stacked GM maize ZDRF8×nCX-1 contains different Bt genes, *cry1Ab* and *cry2Ab* and different herbicide tolerance genes, *g10evo-epsps*, *cp4 epsps*, and *P450-N-Z1*. Since Bt genes are not naturally present in plants, they do not have any known metabolic activity. Glyphosate tolerance is provided by the *g10evo-epsps* and *cp4 epsps* genes. Glyphosate is a potent inhibitor of EPSPS, an important enzyme in the shikimate pathway, which is essential for the biosynthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan.[37] Flazasulfuron and MCPA tolerance is conferred by the cytochrome P450 gene, *P450-N-Z1*. Flazasulfuron controls weeds by inhibiting acetolactate synthase (ALS), which is a necessary enzyme for the production of three branched-chain amino acids, isoleucine, leucine, and valine.[38] MCPA is a selective, systemic hormone-type herbicide that mimics natural growth hormones, leading to uncontrolled cell growth and division, eventually kills the plants.[39] Therefore, there is no need to test for gene interactions between Bt proteins, EPSPS proteins and P450 protein, because they do not share a physiological relationship, do not operate in the same metabolic pathway, and do not exchange any metabolites. Nevertheless, due to the varying pest control spectra

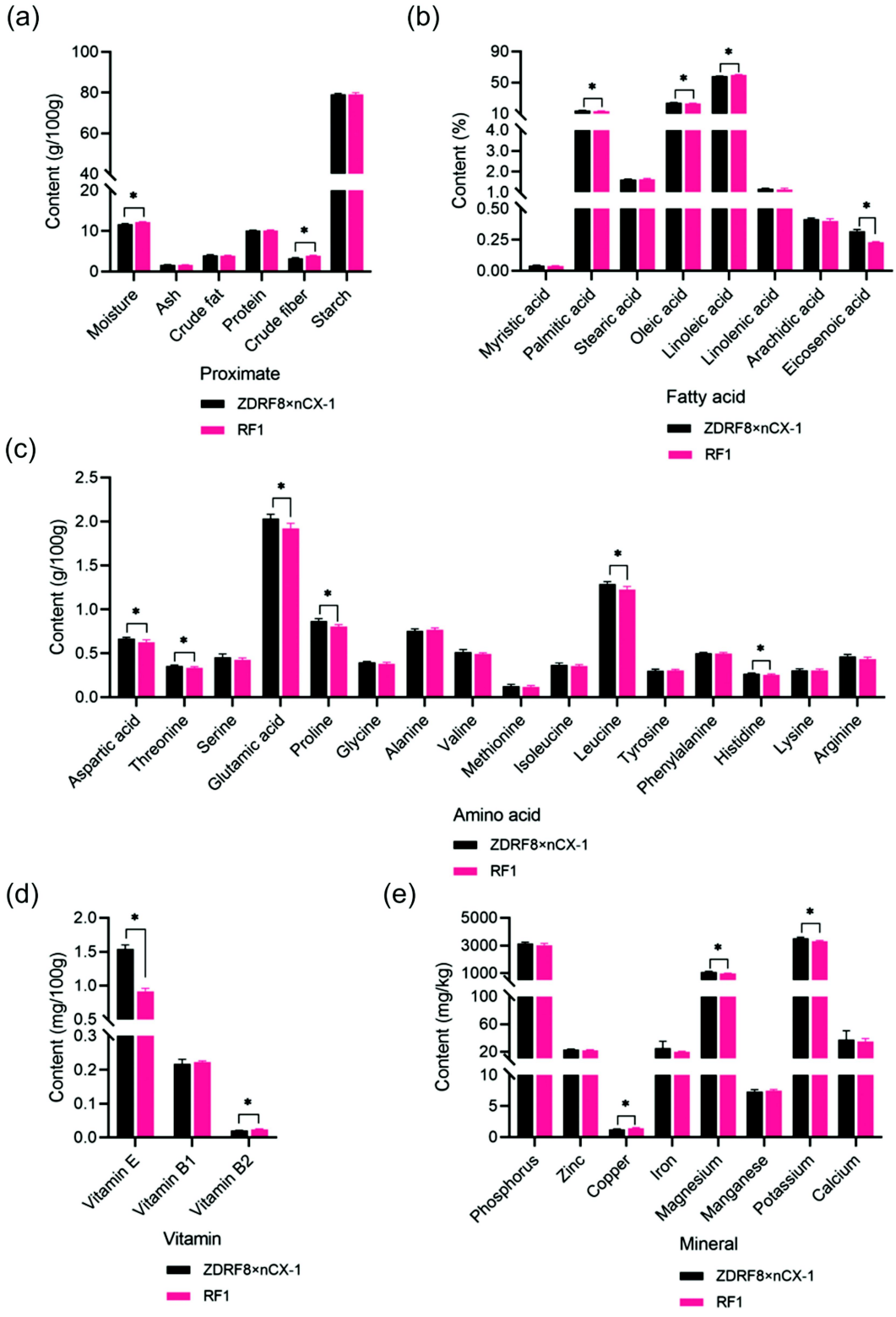

Figure 8. Nutrient composition values of ZDRF8×nCX-1 and non-transgenic maize RF1. Data are presented as mean ± standard deviation ($n = 3$). *A significant difference (adjusted $p < 0.05$) was observed between ZDRF8×nCX-1 and RF1.

of Bt genes, there may be antagonistic or synergistic effects between them. In this study, the amino acid sequences of Cry1Ab and Cry2Ab have lower similarity and they are unlikely to enable cross-resistance. Liang et al. also found that Cry2Ab-resistant *Helicoverpa armigera* exhibited low cross-

resistance to Cry1Ab.[40] The EPSPS enzyme family is ubiquitous in plants and microorganisms. The *g10evo-epsps* and *cp4 epsps* genes were isolated from *Deinococcus radiodurans* and *Agrobacterium* sp. CP4, respectively. The G10evo-EPSPS and CP4 EPSPS proteins are functionally equivalent to endogenous plant EPSPS enzymes, except for their reduced affinity for glyphosate. Therefore, future studies should investigate potential interactions between G10evo-EPSPS and CP4 EPSPS proteins.

Omics techniques are valuable tools for assessing the unintended effects of GM crops. Previous studies have suggested that environmental conditions and genetic background have a greater impact than gene modification.[17,18,41,42] To explore the potential unintended effects of combining transgenes through conventional breeding, the transcriptome profiles of stacked GM maize ZDRF8×nCX-1 leaves expressing traits related to insect resistance and herbicide tolerance were compared to its parent GM maize and isogenic control maize, which were grown in the same environment. A total of 467–1085 DEGs were identified among the four maize lines. The number of DEGs between RF1/ZDRF8×nCX-1, RF1/nCX-1, RF1/ZDRF8 were 615, 1085 and 467, respectively. There were 558 and 882 DEGs between ZDRF8×nCX-1 and its parents ZDRF8 and nCX-1, respectively. Compared to the parent GM maize lines ZDRF8 and nCX-1, the gene-stacked maize ZDRF8×nCX-1 did not exhibit significantly greater effects on transcriptome profiles. In addition, we found the relative expression levels of the P450 family genes in GM maize with the PH4CV background were different from those with the RF1 background. These results indicated that genetic modification and genetic background have a more pronounced impact on the maize transcriptome than hybrid breeding.

Substantial equivalence is a key concept in the safety evaluations of transgenic crops. Across the globe, substantial equivalence has been extensively employed in the safety evaluations of transgenic crops.[43] The stacked-trait transgenic crops created through traditional breeding are also evaluated under the principle of substantial equivalence. Using single-trait transgenic crops as parents to create stacked-trait transgenic crops is essentially the same as traditional cross-breeding, which does not involve molecular-level genetic modification.[44] Safety evaluations are not required for new varieties derived from traditional cross-breeding based on native crop varieties, as they have a long and safe history of use as food or feed. Except for the use of transgenic crops as parents, the traditional cross-breeding method and the development of stacked trait via conventional breeding are equivalent. Also, to guarantee their safety equivalent to that of recipient varieties, parent transgenic crops have undergone extensive safety evaluations. Therefore, it can be concluded that stacked-trait transgenic crops produced through conventional breeding do not pose greater risks to food and feed safety compared to their parental lines.[45,46] Crop composition analysis is a central component of comparative assessment. Based on the nutritional composition analysis of the stacked transgenic maize ZDRF8×nCX-1, the levels of nutrient components were comparable to the non-transgenic counterparts. Similar results were found for the transgenic parents, ZDRF8 and nCX-1.[34,35] These results are consistent with previous studies that stacked GM events do not differ significantly from single GM or non-GM events in their compositional profiles.[47–50]

Conclusion

The conclusion of this work is that the composition of ZDRF8×nCX-1 maize grain is substantially equivalent to that of its non-transgenic comparator. Furthermore, stacking genes through conventional breeding does not cause obvious unintended effects, aside from providing the intended beneficial traits.

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X.Y. and P.W. designed the experiments; X.Y. and H. G. performed the experiments and analyzed the data; X. Y. wrote the draft; P.W. edited this article and provided helpful comments. All authors read and approved the manuscript.

Author contributions

CRediT: **Xiaoxing Yu:** Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft; **Hongyu Gao:** Investigation; **Pengfei Wang:** Conceptualization, Writing – review & editing.

Disclosure Statement

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