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RESEARCH ARTICLE

Unintended changes in transgenic maize cause no nontarget effects

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Societal Impact Statement

Genetic engineering is used to introduce new genes into plants to obtain crops with novel traits. However, the unintended effects of genetically engineered (GE) crops on nontarget organisms—such as beneficial insects—are a topic of much concern. We evaluate the potential unintended effects of pollen from three GE maize and seven commercialized conventional maize lines on the ladybird beetle *Propylea japonica*, by combining omics approaches with feeding assays. Our results suggest that unintended changes caused by genetic engineering in maize pollen may not lead to biologically relevant effects on *P. japonica*. Meanwhile our study provides a useful strategy to assess the biological impacts of genetic engineering on nontarget organisms.

Summary

- The potential effects caused by the inserted traits in genetically engineered (GE) plants on nontarget organisms (NTOs) have been well assessed. However, whether the process of genetic engineering itself causes unintended changes that go beyond the natural variation of the crop and further poses any biological effects to NTOs is still under debate.
- Here, we evaluated the potential unintended effects of pollen from three GE maize and seven commercialized conventional maize lines on the NTO *Propylea japonica* by combining omics approaches with feeding assays.
- The results showed that genetic breeding indeed brought somewhat differences at both proteome and metabolome levels in maize pollen, although such differences were far more common in conventionally crossbred plants. Feeding experiments indicated that the changes in proteins and metabolites caused by genetic breeding did not lead to unintended effects on the NTOs that go beyond those measured for the conventional crossbred lines.

Yuanyuan Wang and Qingsong Liu contributed equally to this work.

- Together, our results suggest that the differences detected by omics experiments may not cause any biological relevant effects on NTOs and the combination of omics approaches and NTOs feeding assays provide a valid approach to assess the biological relevance of compositional effects caused by genetic breeding.

KEYWORDS
Bt maize, metabolome, *Propylea japonica*, proteome, unintended effect

1 | INTRODUCTION

Genetic engineering (GE) is widely used to introduce new genes into plants to obtain crops that exhibit novel traits such as insect or disease resistance, herbicide tolerance, drought tolerance, or improved nutrition (ISAAA (The International Service for the Acquisition of Agri-biotech Applications), 2019; Li et al., 2020). In 2019, a total of 190.4 million hectares of GE crops, increased by approximately 112 times compared with 1996, have been planted in 24 low- and middle-income countries and five industrialized countries worldwide, whereas 42 countries imported GE crops for food, feed, or processing (ISAAA, 2019).

New GE crops must undergo a series of assessments to ensure that they do not pose any unacceptable risk to the environment or to human and animal health before being commercially released (Li et al., 2017; Romeis et al., 2008). Though the focus of the assessment has been on the inserted traits (Ladics et al., 2015; Li et al., 2020), the process of GE may cause the breakage, insertion, activation, or silence of genes inherent in the genome of the recipient plant, and thereby lead to changes in the levels and composition of proteins or metabolites, which may result in unintended effects on nontarget organisms (NTOs) (Ladics et al., 2015; Schnell et al., 2015). The guidelines of environmental risk assessment (ERA) of GE plants issued by European Food Safety Authority (EFSA) thus require to assess such potential unintended effects on valued NTOs (Devos et al., 2016; EFSA, 2010). In fact, effects on certain NTOs have been observed when using plant materials as test substance in feeding assays with GE plants that produce insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) (Li et al., 2015; Liu, Hallerman, et al., 2016; Mason et al., 2008; Romeis et al., 2019; Wang et al., 2017). For instance, in the ERA of Bt crops to the ladybird beetle *Propylea japonica* (Coleoptera: Coccinellidae), a common and abundant natural predator in crop fields, it was found that the larval developmental time of *P. japonica* was significantly prolonged when fed Bt rice pollen containing Cry1C or Cry2A compared with pollen from the non-Bt parental rice (Li et al., 2015). But another study reported that the larval developmental time of *P. japonica* was significantly shorter when they fed pollen from Bt maize than the non-transformed counterpart pollen (Liu, Liu, et al., 2016). Likewise, Wang et al. (2017) reported that there was significant difference in nymphal duration and fecundity of the brown planthopper *Nilaparvata lugens* (Hemiptera: Delphacidae) fed on Bt rice KMD2 producing Cry1Ab compared with the non-Bt parental line. Although it has been speculated that such effects were attributed to unintended changes in the composition of the GE plant materials rather than the inserted insecticidal proteins, no further studies were conducted.

In recent decades, omics technologies have been established and used to investigate the unintended effects of GE crops at the transcriptome, proteome, and metabolome levels (Barros et al., 2010; Frank et al., 2012; Fu et al., 2019; Gayen et al., 2016; Kogel et al., 2010; Liu et al., 2020; NASEM (National Academies of Sciences, Engineering, and Medicine), 2016; Raybould & Macdonald, 2018; Wang et al., 2018). In most cases, the comparisons were limited to one or few crop lines, and the results indicated that there were relatively few differences between the GE lines and their non-GE counterparts. However, whether the process of GE causes such unintended changes that go beyond the natural variation of the crop and further poses any biological effects to NTOs is still vague due to limitations of conventional NTO feeding assays that typically tested only one GE line compared with their counterparts. The application of omics technologies may provide a new strategy to link the unintended effects to the results from feeding assays with NTOs (EFSA, 2010).

Maize (*Zea mays* L.), one of the most widely grown cereals in China, is severely attacked by more than 200 pests from several insect orders, including Lepidoptera (*Ostrinia furnacalis* Guenee, *Conogethes punctiferalis* Guenee, *Mythimna separata* Walker), Coleoptera (*Monolepta hieroglyphica* Motschulsky), and Aphidoidea (*Rhopalosiphum maidis* Fitch), which significantly impede the increase of maize yield (Li et al., 2020; Liu, Hallerman, et al., 2016). To control those pests, millions of tons of broad-spectrum chemical insecticides have been applied, causing serious problems, such as environmental pollution, food contamination, the evolution of insecticide resistance in the targets, and ecological unbalance. In recent years, China devoted great efforts in the development of GE maize varieties against insect pests, and dozens of GE maize lines expressing Bt proteins have been developed. Laboratory and field experiments have shown that most of these lines exhibited effective control of the target lepidopteran pests, making them a viable pest control method (Li et al., 2020; Liu, Hallerman, et al., 2016).

Here, we made the first attempt to assess the potential unintended effects of GE maize pollen on the ladybird beetle *P. japonica* by combining omics technologies with insect feeding assays. In addition, comparisons were not limited to three GE lines and their

TABLE 1 Information on the 10 maize lines used in the current study

Maize line	Abbreviation	Inserted genes	Source	Designated in this study
Zhongdan121	**ZD121**	–	Chinese Academy of Agricultural Sciences	Parental line of CM121
CM8101-121	**CM121**	*cry1Ab-Ma*	Chinese Academy of Agricultural Sciences	Genetic engineering line
Zhengdan958	**ZD958**	–	Chinese Academy of Agricultural Sciences	Parental line of CM958
CM8101–958	**CM958**	*cry1Ab-Ma*	Chinese Academy of Agricultural Sciences	Genetic engineering line
Lianchuang303	**LC303**	–	Zhejiang University	Parental line of SK12-5
Shuangkang12-5	**SK12-5**	*cry1Ab/cry2A* + *G10evo-epsps*	Zhejiang University	Genetic engineering line
Aoyu116	**AY116**	–	Origin Agritech Limited	Conventional maize line
Zhongnongda208	**ZND208**	–	China Agricultural University	Conventional maize line
Zhongnongda209	**ZND209**	–	China Agricultural University	Conventional maize line
Nonghua106	**NH106**	–	DBN Biotech Center	Conventional maize line

respective non-GE parental lines, but also included four additional conventional hybrid maize lines. The total of seven non-GE maize lines thus provided a range of natural variation in the analyzed parameters and allowed interpreting the potential relevance of observed differences between GE and non-GE parental lines.

2 | MATERIALS AND METHODS

2.1 | Pollen preparation

Ten maize lines were used in this study, including three GE maize lines and seven conventionally hybrid maize lines (Table 1). The GE maize line Shuangkang 12-5 (SK12-5) expresses a *cry1Ab/cry2A* fusion gene driven by the maize ubiquitin promoter, linked in tandem with an *EPSPS* (5-enol-pyruvylshikmate-3-phosphate synthase) gene, and its corresponding non-transformed near isoline Lianchuang303 (LC303) (Chang et al., 2017). The GE maize lines CM8101-121(CM121) and CM8101-958(CM958) both express *cry1Ab-Ma* gene redesigned from the *cry1Ab* gene. All GE maize lines were developed through the *Agrobacterium*-mediated method and have high efficacy against lepidopteran pests (Chang et al., 2017; Zhao et al., 2002).

All maize seeds were sown simultaneously in the experimental field station of Jilin Academy of Agricultural Sciences (Northeast Agricultural Research Center of China) in Gongzhuling City, Jilin Province, China (43°19′N, 124°29′W). The maize plants were grown in adjacent plots (plot size: 10 × 5 m) and cultivated equally according to the common local agricultural practice in 2018. All maize plants were thus grown under the same environmental and agriculture conditions. For each maize line, three plots were planted. During maize anthesis in late July 2018, pollen was collected by shaking the tassels into pollination bags (11 × 22 cm) and subsequently passed through a screen (0.125-mm openings) to remove anthers and contaminants. Pollen of 10 maize plants were collected and pooled together as one biological replicate, and three biological replicates were collected for each line for omics profiling. The sieved pollen was kept in cryovials and stored in liquid nitrogen immediately in the field. And then all samples were transferred and stored in −70°C for further analyses.

2.2 | Insects

Adults of *P. japonica* were collected in non-GE maize fields at the experimental field station of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (CAAS), Langfang City, Hebei Province, China (39°5′N, 116°7′E) in 2018. The colony was subsequently maintained in the laboratory referred to our previous studies (Liu, Liu, et al., 2016; Zhang et al., 2014). Newly hatched larvae of *P. japonica* (<12 h after emergence) were used in the bioassays.

2.3 | Proteome profiling

2.3.1 | Protein preparation

A lysis buffer 3, 1 mM PMSF (phenylmethanesulfonyl fluoride), 2 mM EDTA (ethylenediaminetetraacetic acid), and DTT (DL-dithiothreitol) with a final concentration of 10 mM were added into each pollen sample. The mixture was oscillated with a tissue grinder for 2 min and then centrifuged at 25,000 × *g* for 20 min at 4°C to remove the impurity. Ten mM DTT was added into the supernatant and heated in a water bath at 56°C for 1 h. After returning to room temperature, the mixture was put in a dark room for 45 min at a final concentration of 55 mM. And then four volumes of cold acetone were added into the protein mixture and set aside at −20°C for 2 h until the supernatant was colorless. The sediment was grind with the lysis buffer 3 for 2 min and then centrifuged at 25,000 × *g* for 20 min at 4°C. Standard BCA assay was used to detect protein concentrations of all samples. Subsequently, 5 μg trypsin enzyme was added to 100 μg protein solution per sample in two portions and digested at 37°C for 4 h at the first time and for 8 h at the second time. The enzymatic peptides were desalted using a Strata X column and dried under vacuum. Ten μg peptide samples per treatment were mixed together, and 200 μg mixtures were diluted with 2 ml Mobile Phase A including 5% acetonitrile (ACN, pH 9.8) and injected into the LC-20AB liquid phase system (Shimadzu, Japan) subjected to liquid phase separation on a Gemini C18 column (4.6 × 250 mm, 5 μm) according to manufacturer's instructions. It was eluted at a flow rate gradient of 1 ml min^{-1}—5%

Mobile Phase B including 95% cerium ammonium nitrate (CAN, pH 9.8) for 10 min, 5%–35% Mobile Phase B for 40 min, 35%–95% Mobile Phase B for 1 min—and lasted for 3 min and then 5% Mobile Phase B equilibrated for 10 min. The elution peak was monitored at a wavelength of 214 nm (Shimadzu, Japan), and one component was collected every minute. Combined with a chromatographic elution peak map, 10 components were obtained and then freeze-dried. Ten components were collected and freeze-dried in a vacuum concentrator.

The dried peptides were reconstituted with Mobile Phase A (0.1% formic acid, FA in 2% CAN) and centrifuged at 20,000 × *g* for 10 min. The supernatant was transferred and separated by ultra-high-performance liquid chromatography (UHPLC, Thermo, UltiMate 3000). Firstly, the samples were enriched desalted in the trap column and then serially connected to a self-loaded C18 column (150 μm inner diameter, 1.8 μm column size, 25 cm column length) at a flow rate of 500 nl min^{-1} through the following effective gradient: 0–5 min, 5% Mobile Phase B (98% CAN, 0.1% FA); 5–160 min, increased from 5% to 35% linearly; 160–170, increased from 35% to 80%; 175 min, 80%; 176–180 min, 5%.

2.3.2 | Mass spectrometry (MS)

The nanoliter liquid-phase separation end is connected directly to the mass spectrometer. The peptides separated by liquid phase were ionized by a nanoESI source and then passed to a tandem mass spectrometer Q-Exactive HF (Thermo Fisher Scientific, San Jose, CA) for data-dependent acquisition (DDA) mode detection and data-independent acquisition (DIA) mode detection.

For DDA mode, the first-order mass spectrometer scan (MS1, mass range 350–1500 m z^{-1}) was obtained at 60,000 resolution and tuned to 15,000 for the secondary mass spectrometer scan (MS2) at 100 m z^{-1}. The fragments were generated using higher energy collision induced dissociation (HCD) and detected in Orbitrap. The dynamic exclusion duration time was 30 s, and the automatic gain control (AGC) was set to Level 1 as 3e6 and Level 2 as 1e5.

DIA analysis was performed using the same system as DDA analysis. The ion source voltage was 1.6 kV; MS1 scan (350–1500 m z^{-1}) was obtained at 120,000 resolution. Sixty windows were used for variable isolation window acquisition.

2.3.3 | Proteomic data and bioinformation analysis

The raw data of DDA were identified using the integrated Andromeda engine of MaxQuant (http://www.maxquant.org, Max Planck Institutes, Germany) (Cox & Matthias, 2008). DIA raw files were analyzed with Spectronaut 5, a mass spectrometer vendor-independent software from Biognosys, and the false discovery rate (FDR) was set to 1% at peptide level (Bruderer et al., 2015). Proteins that met the expression fold change (FC) ≥ 2 and *P*-value < 0.05 (two-sided unpaired Student's *t*-test) using MSstats package were filtered for the following analyses (Bruderer et al., 2015; Choi et al., 2014). Pathway mapping and function annotation of the DEPs were performed by utilizing the NCBI NR (RefSeq non-redundant proteins) database (https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/), Swiss-Prot database (https://www.uniprot.org/), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (https://www.genome.jp/kegg/pathway.html, Version 84).

2.4 | Metabolite profiling

2.4.1 | Metabolite extraction

Eight hundred μl frozen methanol mixed with water at a ratio of 1:1 was added into each sample. After ground and centrifuged, 400 μl supernatant were transferred and lyophilized, and then 400 μl Solution A was added to reconstitute. Before detection, the solid-phase extraction (SPE) column (strata) was activated and equilibrated using 1 ml 100% acetonitrile, 1 ml 100% methanol, and 1 ml Milli-Q integral purified water. Then 300 μl supernatant was added to the activated SPE column, and a positive pressure extractor was used to carry the sample slowly through the column. The precipitation was washed using 30% acetonitrile twice and 300 μl 100% acetonitrile once.

2.4.2 | Data acquisition

Data acquisitions were performed using a liquid chromatography–mass spectrometry (LC-MS) system. Firstly, all chromatographic separations were performed using an UPLC system (2777C UPLC, Waters, USA). An ACQUITY UPLC BEP C18 column (100 × 2.1 mm, 1.7 μm, Waters, USA) was used for the reversed-phase separation. The column oven was maintained at 50°C. The flow rate was 0.4 ml min^{-1}, and the mobile phase consisted of Solvent A (water + 0.1% FA) and Solvent B (acetonitrile + 0.1% FA). Gradient elution conditions were set as follows: 0–2 min, 100% Phase A; 2–11 min, 0%–100% Phase B; 11–13 min, 100% Phase B; 13–15 min, 0%–100% Phase A.

A high-resolution tandem mass spectrometer SYNAPT G2 XS QTOF (Waters, USA) was used to detect metabolites eluted form the column. The Q-TOF was operated in both positive and negative ion modes. For positive mode, the capillary and sampling cone voltages were set at 2 kV and 40 V, respectively, as well as negative mode. The mass spectrometry data were acquired in Centroid MSE mode. The TOF mass range was from 50 to 1200 Da, and the scan time was 0.2 s. For the MS/MS detection, all precursors were fragmented using 20–40 eV scanning for 0.2 s. During the acquisition, the LE signal was acquired every 3 s to calibrate the mass accuracy. Furthermore, in order to evaluate the stability of the LC-MS during the whole acquisition, a quality control (QC) sample (pool of all samples) was acquired after every 10 samples.

2.4.3 | Metabolite data analysis

The peaks were extracted from the raw data using business software Progenesis QI (Version 2.2, Waters, USA). MetaboAnalyst 4.0 (Chong et al., 2018), AgriGO toolkits (Tian et al., 2017), and the Plant Gene Set Enrichment Analysis Toolkit (PlantGSEA) (Yi et al., 2013) were used for the PCA analysis, Gene Ontology enrichment analysis, and KEGG analysis, respectively.

2.5 | Feeding bioassay with *P. japonica*

In this study, a pollen-based diet was used to conduct the bioassay, which was developed and validated to assess the potential effects of GE maize pollen on *P. japonica* larvae (Liu, Liu, et al., 2016; Zhang et al., 2014). In brief, newly hatched larvae (<12 h after emergence) were individually kept in Petri dishes (6.0 cm diameter, 1.5 cm height). They were fed with maize pollen on the first day of each instar and then provided a mixture of pollen and soybean aphids until development into the next instar. The pollen was directly sprinkled on the bottom of the Petri dish, whereas the aphids were provided on 1-cm segments of heavily infested soybean seedlings. Pollen was replaced every other day, and aphids were replaced daily. In addition, an open 1.5 ml centrifuge tube containing solidified 1% agar solution was added to each dish as a water source. All food elements were provided ad libitum.

Thirty neonates of *P. japonica* were tested for each maize lines. Eclosion rate and developmental time (from larval hatching to adult emergence) were recorded based on daily observation. When adults emerged, females and males were separately weighted using an electronic balance (CPA225D, Sartorius AG, readability = 0.1 mg, repeatability ± 0.1 mg). Experiments were conducted in climate chambers at 26 ± 1°C, 75 ± 5% RH, and a 16L: 8D photoperiod.

2.5.1 | Statistical analysis

One-way ANOVA followed by Tukey HSD test was used to compare the parameter of female and male adult weight. Kruskal–Wallis and chi-square tests were performed, respectively, to compare the developmental times (from larval hatching to adult emergence) and eclosion rates. All analyses were conducted with the software package SPSS (Version 13 for Windows).

3 | RESULTS

3.1 | Proteomics analysis

Via DDA analysis for all maize pollen treatments, there were a total 43,930 peptides and 6979 proteins detected. Specifically, the protein coverage of 93.6% of all the proteins distributed between 0% and 50%, with the largest number of proteins in the 10%–20% interval (Figure S1a). About 22.3% of proteins contained one unique peptide, and 19.2% for two unique peptides (Figure S1b). The protein mass of 71.4% of total proteins ranged from 10 to 60 kDa, with 16.2% in the interval 20–30 kDa, 16.0% in 30–40 kDa, and 15.3% in 40–50 kDa (Figure S1c). The DDA experiment raw data files were imported to MaxQuant software packages (https://www.maxquant.org/) for DIA analysis.

For the proteome profiling, heatmap cluster analyses of the total detected proteins showed that GE maize CM121, CM958, and SK12-5 clustered together with their corresponding parental lines ZD121, ZD958, and LC303, respectively (Figure 1). Among the four commercial non-GE lines, AY116, ZND208, and ZND209 clustered as a group and clearly separated from NH106 (Figure 1). Subsequently, the differentially expressed proteins (DEPs) between GE and non-GE lines or between any two conventional breeding lines were screened, taken FC ≥ 2 and *P*-value < 0.05 as the condition. The number of DEPs detected in GE/parental maize pairs ranged from 5 to 94 and was thus comparable or lower than the DEPs detected for any of the pairs of non-GE maize lines (ranging from 24 to 624) (Figure 2a). Specifically, the numbers of DEPs in the comparisons between GE and their corresponding parent varieties were 5 for CM958/ZD958 (3 upregulated, 2 downregulated), 31 for CM121/ZD121 (13 upregulated, 18 downregulated), and 94 for SK12-5/LC303 (30 upregulated, 64 downregulated), respectively (Figure 2a). The Venn diagrams presented the distribution of DEPs for each GE/non-GE comparison (Figure 2b). The DEPs of the comparison between any two conventional crossbred maize lines were pooled together as one group. Among all comparisons, a total of 1457 DEPs were identified, of which 126 DEPs in summary for all comparisons between GE and their corresponding parent lines. Specifically, more than 80.5% DEPs detected in the GE/parental comparisons were also observed in the comparisons between conventional lines (Figure 2b). There were only 11 DEPs detected in the comparison between CM121 and ZD121 and 14 DEPs detected between SK12-5 and LC303 that were not found in any of the non-GE maize comparisons, and no unique DEPs were observed between CM958 and ZD958 (Figure 2b). However, these 24 DEPs seem to commonly exist in maize tissues as indicated by comparing with protein sequences in the NCBI database (https://www.ncbi.nlm.nih.gov/) (Dataset S1).

3.2 | Functional enrichment analyses of DEPs

KEGG pathway enrichment analyses were performed to explore the biological function of DEPs in the 24 pairwise group comparisons. In Conventional/Conventional line comparisons, the DEPs enriched significantly in 132 different pathways, whereas 20 pathways were significantly enriched in the three GE/non-GE pairwise group comparisons. Specifically, the Top 5 of all pathways that the DEPs mainly enriched significantly in Conventional/Conventional line comparisons were metabolic pathway (ko01100), biosynthesis of secondary metabolites (ko01110), biosynthesis of amino acids (ko01230), carbon metabolism (ko01200), protein processing in endoplasmic reticulum (ko04141) (Dataset S2).

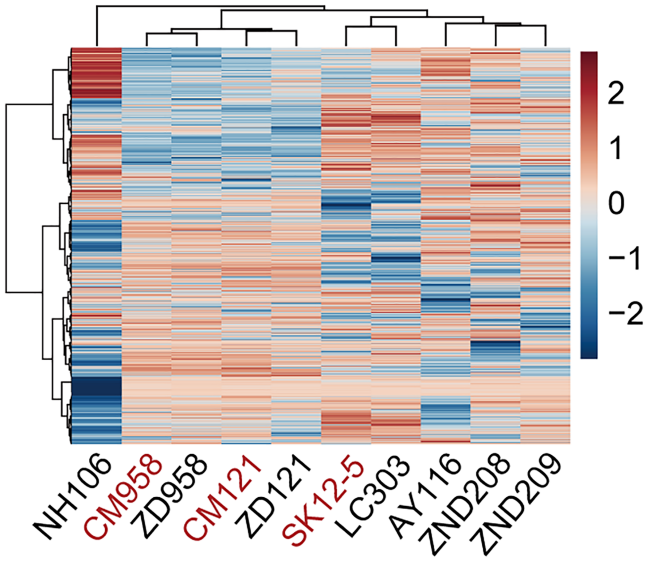

FIGURE 1 Hierarchical clustering of 10 maize lines using the total detected protein data. In the heatmap, each maize line is visualized in a single column, and each protein is represented by a single row. Protein expression levels are shown in different colors, where red indicates high abundance and blue indicates low abundance (color key scale right of the heatmap). Genetically engineered (GE) maize lines and non-GE maize lines are labeled in red and black, respectively, in the axis legends

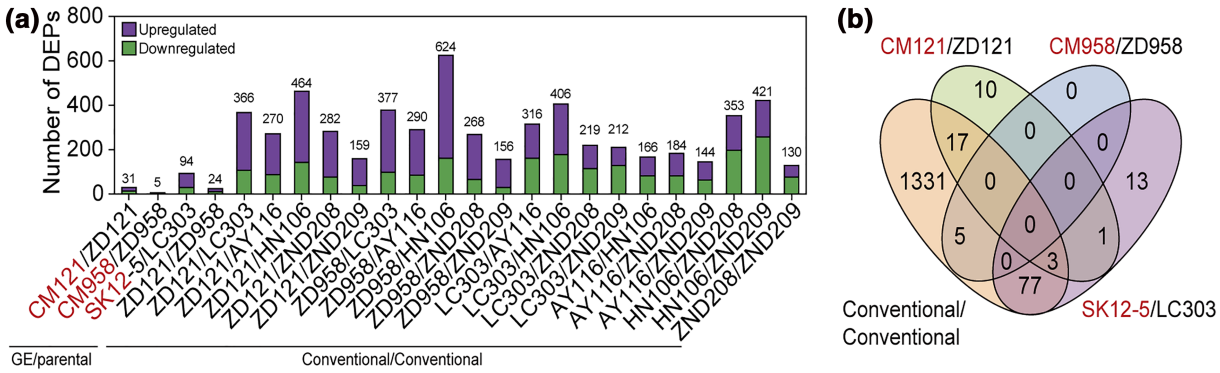

FIGURE 2 Differentially expressed proteins (DEPs) among the tested 10 maize pollen. (a) Pairwise comparisons of differentially expressed proteins (DEPs) between different maize lines. Pairwise comparisons were conducted between genetically engineered (GE) lines and their non-GE parental counterparts and between any two non-GE maize lines. (b) Venn diagrams depicting the unique and shared differentially expressed proteins (DEPs) among conventional maize lines and GE lines. Genetically engineered (GE) maize lines and non-GE maize lines are labeled in red and black, respectively, in the axis legends

For GE/non-GE comparison groups, four DEPs in CM121/ZD121 involved into six KEGG pathways, including biosynthesis of secondary metabolites (ko01100), carotenoid biosynthesis (ko00906), endocytosis (ko04144), metabolic pathways (ko01100), proteasome (ko03050), and starch and sucrose metabolism (ko00500), whereas eight DEPs in SK12-5/LC303 involved in 17 KEGG pathways, of which the Top 5 were metabolic pathways (ko01100), biosynthesis of secondary metabolites (ko01110), protein processing in endoplasmic reticulum (ko04141), RNA transport (ko03013), and sphingolipid metabolism (ko00600) (Dataset S2). All these pathways involved in GE/non-GE line groups could also be found in Conventional/Conventional line comparisons.

3.3 | Metabolomics analysis

Untargeted metabolomics analysis was used to profile the metabolites in pollen of all 10 maize varieties. In total, 9411 and 9567 ion features were detected under positive (POS) and negative (NEG) electrospray ionization (ESI) mode, respectively. After low-quality ion filtering, 8090 and 7403 ion features in respective modes were left that satisfied relative standard deviation (RSD) ≤ 30% in all QC samples for further analysis.

To investigate the difference of metabolites in pollen of all different maize lines, a principal component analysis (PCA) was performed on all detected ion features under each mode to obtain a global view

of the metabolites across the 10 maize lines. PCA revealed a clear separation among maize lines (ZND208, LC303, and SK12-5 vs. ZD121, CM121, ZD958, CM958, ZND209, AY116, and NH106), but no separation of GE and parental lines under both POS and NEG modes (Figure 3a,d). The total number of differentially accumulated metabolic features (DAMFs) under both POS and NEG modes in GE/parental comparisons ranged from 0 to 80 and were much less common when compared with those in the pairwise comparisons among the seven non-GE lines (ranging from 0 to 3462) (Figure 3b,e). The non-GE group LC303/NH106 had the highest DAMFs under both POS and NEG modes, whereas no signature metabolite was detected in two non-GE group (ZD121/AY116, ZD958/ZND209) and one GE/parental comparison (CM958/ZD958) (Figure 3b,e). All 12 unique DAMFs were detected in the SK12-5/LC303 comparison under NEG modes (Figure 3c,f).

3.4 | Feeding bioassay with *P. japonica*

A feeding bioassay was carried out to determine whether the differences detected in pollen proteome and metabolome pose any effect of biological relevance to the NTO *P. japonica*. Across all maize lines tested, the eclosion rate varied between 63.3% and 90% (Figure 4a), the developmental time (time to eclosion) from 12.95 to 13.71 days (Figure 4b), male fresh weight from 4.81 to 5.75 mg (Figure 4c), and female fresh weight from 5.81 to 6.50 mg (Figure 4d). The statistical analyses revealed no differences for any of the recorded parameters between any of the tested GE or non-GE maize lines (All $P > 0.05$).

4 | DISCUSSION

The occurrence of unintended effects caused by the process of GE and their impact on NTOs are a concern when deploying GE crops (Devos et al., 2016; Ladics et al., 2015; Liu et al., 2020; NASEM, 2016; Ricroch et al., 2011). In this study, we integrated proteomics and metabolomics analysis with insect feeding assays and demonstrated that unintended changes caused by GE in maize did not cause any adverse effects on the nontarget *P. japonica*.

In some previous studies, effects of GE plants on NTOs were reported when material from Bt plants was used as test substance (Li et al., 2015; Liu, Liu, et al., 2016; Romeis et al., 2013, 2019). The relevance of the observed effects was generally difficult to interpret as typically only one GE plant was compared with the closest related non-GE counterpart. Therefore, it was suggested to incorporate the natural variation among tested crop lines to interpret the differences between GE lines and non-GE comparator (Chen et al., 2021a, 2021b; Liu et al., 2020; Meissle et al., 2014; NASEM, 2016; Wang et al., 2018, 2019). In the present study, 10 maize lines including three GE maize lines containing insecticidal and/or herbicide tolerance genes and their non-GE parental lines as well as four conventional commercialized maize lines were used. We thus

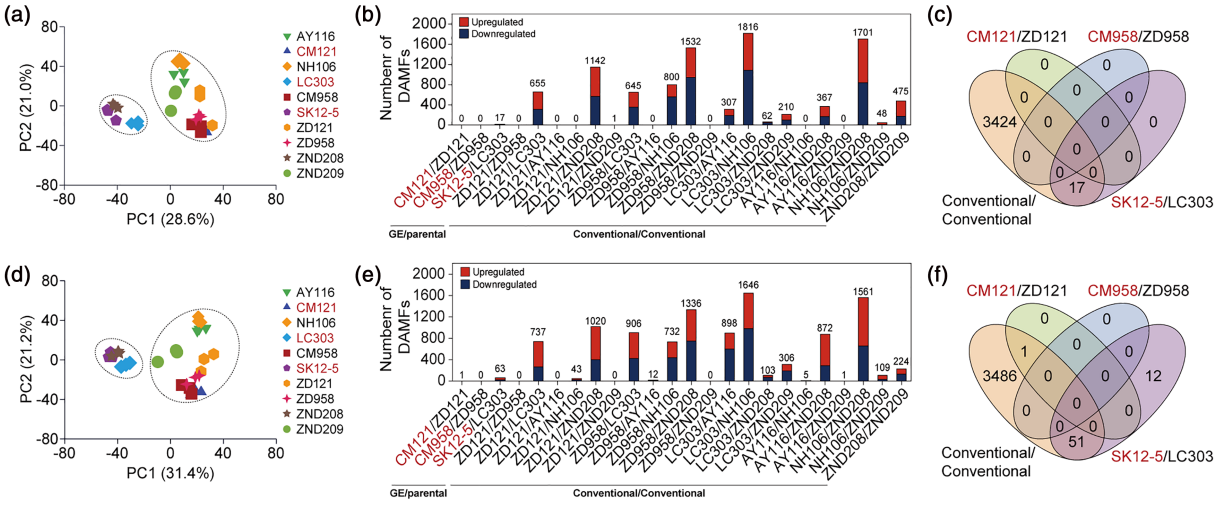

FIGURE 3 Metabolome analysis of the 10 maize pollen. (a) Principal component analyses (PCA) of metabolite accumulation levels in pollen of 10 maize lines under positive mode. Score plot of the first two principal components with the explained variance. (b) Pairwise comparisons of differentially accumulated metabolic features (DAMFs) between different maize lines under positive mode. Pairwise comparisons were conducted between GE lines and their corresponding non-GE parental lines and between any two conventional hybrid maize lines. (c) Venn diagrams depicting the unique and shared DAMFs among conventional maize lines and GE lines under positive mode. (d) PCA of metabolite accumulation levels in pollen from 10 maize lines under negative mode. Score plot showed the first two principal components with the explained variance. (e) Pairwise comparisons of DAMFs between different maize lines under negative mode. Pairwise comparisons were conducted between GE lines and their corresponding non-GE comparators and between any two conventional hybrid maize lines. (f) Venn diagrams depicting the unique and shared DAMFs among conventional maize lines and GE lines under negative mode. Genetically engineered (GE) maize lines and non-GE maize lines are labeled in red and black, respectively, in the axis legends. Samples clustered closer together were marked with gray rings

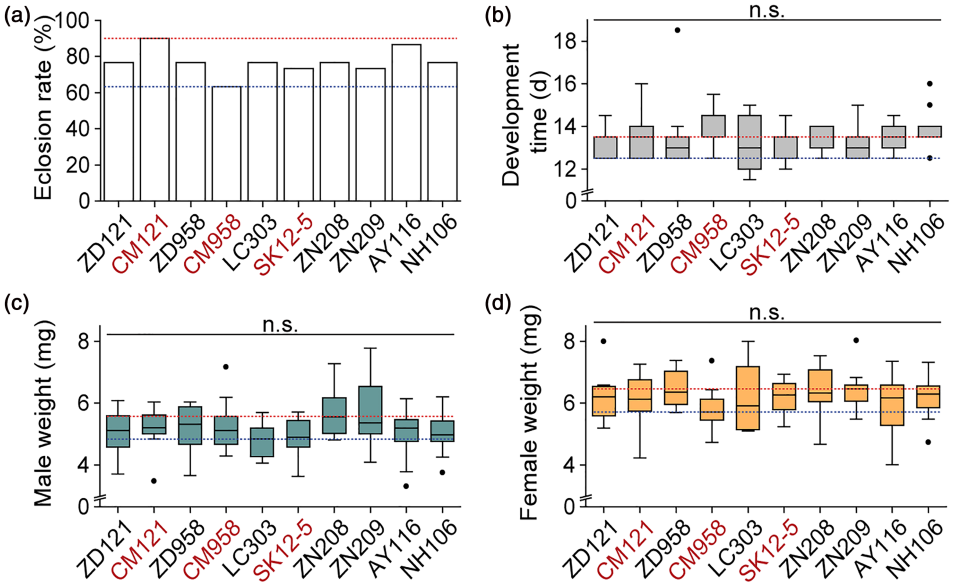

FIGURE 4 Performance of *Propylea japonica* fed pollen from genetically engineered (GE) maize or the conventional maize lines. (a) Eclosion rate of *P. japonica* fed pollen from 10 maize lines ($n = 30$). (b) Developmental time (larval hatching to adult emergence) of *P. japonica* larvae fed pollen from GE maize or the conventional maize lines ($n = 30$). (c) Male weight of *P. japonica* adults developed from larvae fed pollen from genetically engineering (GE) maize or the conventional maize lines ($n = 9$–15). (d) Female weight of *P. japonica* adults developed from larvae fed pollen from GE maize or the conventional maize lines ($n = 8$–17). The parameters of development time and eclosion rates were analyzed using Kruskal–Wallis and chi-square tests, respectively. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant differences (HSD) test was carried out for the weight of female and male adults. n.s. indicates a nonsignificant difference ($P > 0.05$). The blue and red dotted lines represent the minimum and maximum values of the mean in the graph (a–d), respectively. The error bars in (b–d) show the standard errors. Genetically engineered (GE) maize lines and non-GE maize lines are labeled in red and black, respectively, in the axis legends

established the natural variation for these tested maize lines within our study, although the natural range of variation for all potential maize lines is likely to be much broader. In addition, maize pollen was chosen as test material in our experiment because pollen is utilized by several arthropods like lacewings, ladybird beetles, and honeybees that can thus be exposed to insecticidal proteins present in GE maize pollen (Duan et al., 2008; Li et al., 2017; Meissle et al., 2014; Romeis et al., 2019).

Published evidence for maize, wheat, soybean, and rice indicate that environmental factors and meteorological conditions such as the growing location and season pose stronger effects on the global gene expression, protein, and metabolite production than the GE process (Barros et al., 2010; Batista et al., 2017; Baudo et al., 2006; Bedair & Glenn, 2020; Cheng et al., 2008; Corujo et al., 2019; Frank et al., 2012; Tan et al., 2019; Wang et al., 2018). We therefore collected pollen from maize plants that were simultaneously cultivated at the same field site and thus differences in the proteome and metabolome of the test lines are likely to reflect the impact of genetic variation. Furthermore, the stacking of different GE events by crossbreeding did not generate novel variation beyond that of traditional breeding (Agapito-Tenfen et al., 2014; Wang et al., 2018). In our study, both proteome and metabolome analyses indicated that there were indeed differences between GE maize lines and their respective non-GE counterparts; however, the majority of the DEPs and DAMFs were also found in the pairwise comparisons among non-GE conventional maize lines that were all commonly commercialized in China and considered as safe. Moreover, the variation in maize pollen brought by genetic breeding was much smaller than that among conventional maize lines, although some unique DEPs and DAMFs were detected. These results are in line with previous studies that reported less variations in gene expression and metabolite accumulation in tissues between GE and non-GE parental lines when compared with the variation from conventional maize hybrids (Wang et al., 2018). Similar results were also found in a study with rice, which revealed a clear discrimination between rice lines with *Geng/Japonica* and those with *Xian/Indica* genetic background, in both transcriptomic and metabolomic levels in leaves, regardless of transgenic breeding or conventional crossbreeding, whereas the detected differentially expressed genes and metabolites when comparing GE rice and the corresponding non-GE rice lines were considerable to those observed when comparing conventional cross-bred rice with their parental rice lines (Liu et al., 2020).

Although only a few differences in both proteins and metabolites between the GE maize lines and their non-GE counterparts were detected, the question remained whether they pose any effects of biological relevance to NTOs. We thus conducted feeding assays with the ladybird beetle *P. japonica*. The results showed that feeding on GE maize pollen had no significant effect on a number of life-table parameters (eclosion rate, developmental time, and adult weight) of *P. japonica* compared with those feeding on non-GE parental maize pollen or pollen from other conventional maize lines. The variation of the

growth parameters of *P. japonica* feeding on pollen from the three GE maize lines was always within the range of variation among the different non-GE lines, indicating that ingestion of GE maize pollen used in the current study has no unintended effects on the performance of this important nontarget species. Similarly, no difference was found when *Chrysoperla carnea* (Neuroptera: Chrysopidae) larvae fed pollen from Bt maize expressing Cry1Ab or Cry3Bb1 protein or pollen from their corresponding non-transformed near isolines or three conventional lines although difference in total protein content, C:N ratio, and grain diameter among all the tested maize pollen were detected (Meissle et al., 2014). Recently, Chen et al. (2021a, 2021b) investigated the performance of *Daphnia magna* (Diplostraca: Cladocera) on flour, leaves, and pollen from two stacked SmartStax maize lines containing six different Bt Cry proteins and a set of non-GE conventional lines and found that nutritional stress and plant background effects can explain differences in *D. magna* performance observed between GE plants and their non-GE comparators.

Taken together, the current study suggests that (i) changes in proteins and metabolites between GE plants and their parental lines detected by omics technologies do not necessarily cause unintended effects on NTOs; (ii) combination of omics analyses and insect feeding assays allows a more comprehensive and explicable evaluation of unintended effects of GE crops on NTOs; and (iii) it is valuable to include a number of conventional non-GE comparators when evaluating unintended effects of GE crops that provide information on the natural variation and thus allow the interpretation of potential effects detected (Chen et al., 2021a, 2021b; Liu et al., 2020; Meissle et al., 2014; Wang et al., 2017).

In conclusion, we did find that genetic breeding does result in differences at both proteome and metabolome levels, though such differences were for more common in conventionally crossbred plants, and thus, the changes in proteins and metabolites caused by genetic breeding do not bring unintended effects on NTOs that go beyond those caused by conventional crossbreeding in maize. Combining omics approaches with NTO feeding assays allows assessing the biological relevance of compositional effects caused by genetic breeding.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Y.H.L. conceived the idea and designed the research. Y.Y.W. and X.Y.S. performed the experiments. Q.S.L. and Y.Y.W. analyzed the data. Y.Y.W., Q.S.L., X.W.Y., L.Z.H., J.R., and Y.H.L. wrote the manuscript. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of the study are available in the Supporting Information of this article.

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