Metabolomic Plasticity in GM and Non-GM Potato Leaves in Response to Aphid Herbivory and Virus Infection

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ABSTRACT: An important aspect of ecological safety of genetically modified (GM) plants is the evaluation of unintended effects on plant−insect interactions. These interactions are to a large extent influenced by the chemical composition of plants. This study uses NMR-based metabolomics to establish a baseline of chemical variation to which differences between a GM potato line and its parent cultivar are compared. The effects of leaf age, virus infection, and aphid herbivory on plant metabolomes were studied. The metabolome of the GM line differed from its parent only in young leaves of noninfected plants. This effect was small when compared to the baseline. Consistently, aphid performance on excised leaves was influenced by leaf age, while no difference in performance was found between GM and non-GM plants. The metabolomic baseline approach is concluded to be a useful tool in ecological safety assessment.

KEYWORDS: *Solanum tuberosum*, genetic modification, risk assessment, potato virus Y, *Myzus persicae*, NMR, metabolomics

■ INTRODUCTION

One of the concerns regarding the cultivation of genetically modified (GM) plants is their possible impact on insect ecology and biodiversity in agricultural fields.[1] Measuring such effects, however, is not straightforward because ecological impacts are neither easily defined nor is their chance of occurrence easily predicted. Fundamental knowledge of complex ecological interactions would often be required, and this knowledge is in most cases not readily available. Comparative risk assessment is an alternative that provides clear criteria for safety without directly predicting ecological processes.[2,3] In comparative risk assessment the changes introduced by genetic modification are compared to a baseline of variation present in the system under study. For example, a change in insect performance on a plant due to genetic modification would be considered safe when that change does not exceed the baseline of variation in insect performance on this plant. Baselines should capture the variability in the agricultural system under study and consist of a selection of relevant factors, e.g., variation among different cultivars of the same plant species, different environmental conditions, locations, etc.

The present study applies the comparative approach to the study of risks regarding ecological interactions between a plant, an insect, and a virus species using a GM potato cultivar and its non-GM counterpart as a case study. Because it is practically impossible to measure all ecological interactions between a plant and its associated insect species in all possible environmental conditions, leaf chemistry of plants is used here as an indicator of possible changes to plant−insect interactions. The strong influence of plant chemical traits on ecological relationships with insects has been shown repeatedly: both primary and secondary plant metabolites have been found to affect food webs over several trophic levels above and below ground.[4−7] Thus, demonstrating chemical equivalence between a GM plant and its comparator(s) with a broad, nontargeted

method may be a global indication for its safety with respect to insect ecology.

Plant chemistry, however, is a plastic trait that varies over space and time, and this plasticity has been shown to play an important role in ecological interactions.[8−10] Therefore, a baseline of variation in plant chemistry needs to be established. In this study, plants were grown in climate chambers and subjected to a set of internal and external factors that are assumed to influence plant chemistry in the field: virus infection (potato virus Y), aphid herbivory (*Myzus persicae*), and leaf age. In order to test to what extent the measured chemical variation can indeed serve as an indicator for changes in plant−insect interactions, we measured the performance of *M. persicae* in a bioassay on leaves.

Using chemical information in ecological risk assessment requires broad, nontargeted metabolomic profiling techniques since no prior knowledge on the nature of possible specific changes is available.[11,12] In the present study, nuclear magnetic resonance (NMR) spectroscopy was chosen due to its broad coverage of compounds. In a risk assessment framework, NMR is of particular value due to the simple sample preparation and its good reproducibility across machines.[13−15] NMR is nondestructive and can therefore be easily combined with other methods that are less broad in terms of compound range but more sensitive to low concentrations. NMR has been previously applied to food classification studies (e.g., ref 16), risk assessment in GM plants (e.g., ref 17), and studies of plant−insect interactions (e.g., refs 11 and 18).

In summary, we ask the following: (a) what is the baseline of variation in potato leaf chemistry in response to internal and external factors such as leaf age, virus infection, and aphid

herbivory; (b) how do chemical changes introduced by genetic modification compare quantitatively to this baseline; (c) how does the measured chemical variation in plants relate to aphid performance on these plants?

■ MATERIALS AND METHODS

Plants. In this study the GM potato cultivar "Modena" (grant no. NRR 30805, AVEBE UA, Foxhol, The Netherlands/BASF Plant Science Co. GmbH) and its non-GM counterpart "Karnico" were used. The genetic modification of "Modena" results in higher relative amylopectin yields in tubers, which is achieved by blocking amylose production with an antisense knock down of the granule-bound starch synthase. All plants were grown from tubers in a growth chamber (16:8 h light:dark photoperiod, light intensity 112.3 ± 18.5 μmol m^{-2} s^{-1}, 23 °C, 70% relative humidity) in 5 L pots, covered in insect-proof gauze sleeves. For testing the effect of potato virus Y (PVY) infection on metabolomic profiles, 6-week-old, PVY-infected plants were compared to healthy control plants of the same age. The effect of aphid–herbivory on plant chemistry was tested by infesting 6-week-old healthy plants with aphids for 3 weeks and taking leaf samples from these 9-week-old infested plants as well as from 9-week-old healthy control plants.

Potato Virus Y Infection Treatment. Potato virus Y (PVY) infection occurred naturally in ca. 25% of both GM and non-GM plants grown from tubers in the laboratory. Infection was presumably acquired during the growing season in the field before tuber harvest. The infection status of all plants in the experiment was determined by both visual inspection for symptoms during plant growth and ELISA antibody tests performed on freeze-dried leaf samples by the Dutch General Inspection Service for agricultural seeds and seed potatoes (NAK). After 6 weeks of growth, leaf samples were taken from eight PVY-infected and eight healthy plants of each cultivar (GM and non-GM). From each plant, one young leaf (first fully grown leaf from top) and one old leaf (third leaf from bottom) was sampled.

Aphid Herbivory Treatment. Several individuals of the peach–potato aphid (M. persicae) were taken from a clonal laboratory population and reared for at least one generation on whole plants of the potato cultivar "Nicola", in order to avoid adaptation to either of the experimental cultivars. Sixteen GM and sixteen non-GM plants that were grown in a climate chamber (see above) were used in the experiment. All of these plants were virus free. One-half of the plants of each cultivar were infested with 20 adult aphids, plants were covered with insect-proof gauze sleeves and populations were allowed to build up for 3 weeks. The other half of the plants of each cultivar was kept aphid free. After this period, young (first fully developed) and old leaves (third leaf from bottom) were sampled from both aphid-infested and aphid-free plants.

Extraction of Plant Material. All sampled leaves were frozen in liquid nitrogen immediately after sampling and stored at −20 °C until analysis. Leaf material was extracted and prepared for NMR analysis according to the protocol of Kim et al.[15] Leaf samples were freeze dried and ground to a fine powder (3 min at 30 Hz) in a mixer mill (MM200, Retsch, Germany). Equal amounts of ground material (30 mg) were transferred into 2 mL centrifuge tubes, and 600 μL of KH$_2$PO$_4$ buffer (90 mM, pH 6.0) in D$_2$O and 600 μL of methanol-d_4 (1:1) were added for extraction. As an internal standard, 0.05% trimethyl silyl propionic acid sodium salt (TMSP; w/w) was used. The mixtures were vortexed, ultrasonicated for 10 min, and centrifuged at 13 000 rpm for 10 min. The supernatants were transferred to a 1.5 mL tube and centrifuged again for 1 min at 13 000 rpm before 700 μL of each extract was transferred to an NMR tube.

NMR Analysis. Spectra of ^{1}H NMR measurements as well as J-resolved, COSY, and HMBC spectra were recorded at 25 °C on a Bruker 600 MHz AVANCE II NMR spectrometer (600.13 MHz proton frequency) equipped with TCI cryoprobe and Z-gradient system. CD$_3$OD was used as an internal lock. For a detailed description of the measurement parameters see ref 16. The resulting spectra were manually phased and baseline corrected and calibrated to the internal standard TMSP at 0.0 ppm using XWIN NMR (version

3.5, Bruker). ^{1}H NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Intensities of spectra were scaled to the intensity of the internal standard (TMSP, 0.05% w/v) and reduced to integrated regions ("buckets") of equal width (0.04) corresponding to the region of δ 0.4–δ 10.0. Residual signals of water and MeOH were excluded from analysis by deleting the respective spectral regions of δ 4.8–δ 4.9 and 3.28–δ 3.34. Structure elucidation of compounds was facilitated by J-resolved, COSY, and HMBC spectra and an in-house reference library of isolated compound spectra. Quantification of specific compounds (α-chaconine and α-solanine) was done by measuring peak heights of the signals corresponding to H-6 protons of the aglycone in MestReNova software (version 6.0.2-5475, Mestrelab Reseach S.L.).

Aphid Performance Bioassay. Variation in chemical profiles of healthy plants caused by genetic modification and leaf age was related to the performance of the peach–potato aphid (M. persicae): First, population growth of aphids during the aphid-induction experiment was measured by counting the number of aphids on the plant after 3 weeks. Instantaneous rates of population increase (r_i) were compared between GM and non-GM plants and between young and old leaves. In a second bioassay the scale of the experiment was reduced from whole plants to excised leaves that were placed on a layer of sterile agar in Petri dishes. Gauze was embedded into Petri-dish lids to allow for air flow, and dishes were sealed with parafilm. Two young leaves (two first fully grown leaves) and two old leaves (third and fourth leaf from the bottom) were excised from 6-week-old plants grown under insect-free conditions in a climate chamber. Sixteen replicate plants were used, and five adult M. persicae individuals were placed on each leaf. The number of offspring per leaf after 5 days was recorded.

Data Analysis. The bucketed metabolomics data were mean centered and standardized (variance = 1) prior to all multivariate analyses. The metabolomic distances between samples and groups of samples were determined by nonparametric MANOVA based on permutation of Euclidean distance matrices.[19] This method is similar to the metabolomic distance method introduced by ref 20, except that no data reduction is performed prior to calculation of distances. Analysis was performed in R version 2.12.1[21] with package "vegan" version 1.17-6[22] using 999 permutations.

Principal component analysis (PCA) was used as an unsupervised method to visualize variability and clustering in the data set. Partial least-squares-discriminant analyses (PLS-DA) is a supervised multivariate analysis technique which maximizes the covariance between the X matrix (^{1}H NMR spectral intensities) and the Y matrix (group information). Although qualitatively the same grouping patterns were found in PLS-DA and PCA, the separation of groups was stronger in PLS-DA. The latter was therefore used to identify the variables (and the corresponding compounds) that were most influential to group separation. Both PCA and PLS-DA were performed with SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden). Components were added only when significant according to the cross-validation function of the software. For ^{1}H NMR data from the PVY infection experiment, a PLS-DA with four significant components explained 60.9% of the total variation. In the aphid herbivory experiment a PLS-DA model with three components explained 73.8% of the total variation in metabolomic data. Relative levels of α-chaconine and α-solanine were compared between treatments by performing ANOVAs on data after square-root transformation. Data obtained from the whole-plant bioassay were tested for a difference in means of instantaneous rates of aphid population increases using Student's t test. Data obtained from bioassays with aphids on excised leaves in Petri dishes were analyzed by fitting a generalized linear model (GLM) with a Poisson distribution and log-link function to the data in R version 2.12.1. The model was compared with reduced models in a stepwise manner in order to determine significance of factors.

■ RESULTS AND DISCUSSION

The presence of a number of common primary metabolites was confirmed by NMR, such as glucose α-glc at δ 5.18 (d, J = 3.5 Hz) and β-glc at δ 4.58 (d, J = 7.9 Hz), sucrose at δ 5.40 (d, J =

3.8 Hz) and δ 4.16 (d, *J* = 8.7 Hz), alanine at δ 1.48 (d, *J* = 7.2 Hz), glutamate at δ 2.40 (m), threonine at δ 1.33 (d, *J* = 6.5 Hz), acetic acid at δ 1.93 (s), fumaric acid at δ 6.56 (s), choline at δ 3.24 (s), cytosine/uracil at δ 5.90 (d, *J* = 8.0 Hz) and δ 7.47 (d, *J* = 8.0 Hz). Among the group of secondary metabolites, which are often species specific in plants, a complex pattern of glycoalkaloid (GA) signals in the methyl region δ 0.8−1.3 was found, corresponding to H-18, H-19, and H-21 of the aglycone.[23] Glycoalkaloids occur in plants of the Solanaceae family and have long been known for their bioactivity.[24,25] The two main glycoalkaloids α-chaconine and α-solanine[25] were identified by alignment with NMR spectra obtained from isolated compounds. NMR peak assignments of glycoalkaloids have also been previously reported in ref 26. In particular, signals corresponding to H-6 of the aglycone part proved characteristic for the distinction between the two alkaloids in the mixture: the respective signal of α-chaconine was shifted downfield at δ 5.16 (s) compared to the signal of α-solanine at δ 5.12 (s). Characteristic compounds detected in the phenolic region (δ 6.0−8.0) were 5-caffeoylquinic acid (chlorogenic acid) at δ 6.36 (d, *J* = 16.0 Hz) and its analogues 3- and 4-caffeoylquinic acid at δ 6.40 (d, *J* = 16.0 Hz) and 6.44 (d, *J* = 16.0 Hz), respectively, as well as the alkaloid trigonelline at δ 9.16 (s), δ 8.86 (m), and δ 8.12 (m).

Chemical Baseline Variation: Leaf Age, Virus Infection, and Aphid Herbivory. In both experiments (PVY

Table 1. Sources of Variation in Leaf Metabolomic Profiles in a Potato Virus Y (PVY) Infection Experiment: Nonparametric MANOVA Based on Euclidean Distances between Samples

source of variation	df	SS	explained variation [%]	F	P
GM	1	558.8	5.38	5.49	0.001
PVY infection	1	1738.3	16.73	17.07	0.001
leaf age	1	2241.9	21.58	22.01	0.001
GM: PVY infection	1	491	4.73	4.82	0.001
GM: leaf age	1	247.9	2.39	2.43	0.020
PVY infection: leaf age	1	424.8	4.09	4.17	0.001
residuals	46	4684.9	45.10		
total	52	10 387.7			

Table 2. Sources of Variation in Leaf Metabolomic Profiles in an Aphid Herbivory (*M. persicae*) Induction Experiment: Nonparametric MANOVA Based on Euclidean Distances between Samples

source of variation	df	SS	explained variation [%]	F	P
aphid herbivory	1	465.9	4.00	4.53	0.012
leaf age	1	5501	47.25	53.49	0.001
aphid herbivory: leaf age	1	636.7	5.47	6.19	0.003
residuals	49	5039.4	43.28		
total	52	11 642.9			

infection and aphid herbivory) leaf age had the biggest effect on chemical profiles. This becomes evident by the amounts of explained variation in metabolomic profiles (Tables 1 and 2) and by the clear separation of young and old leaves in PLS-DA score plots along the first component (Figure 1). The most influential variables in PLS-DA causing this age effect in both
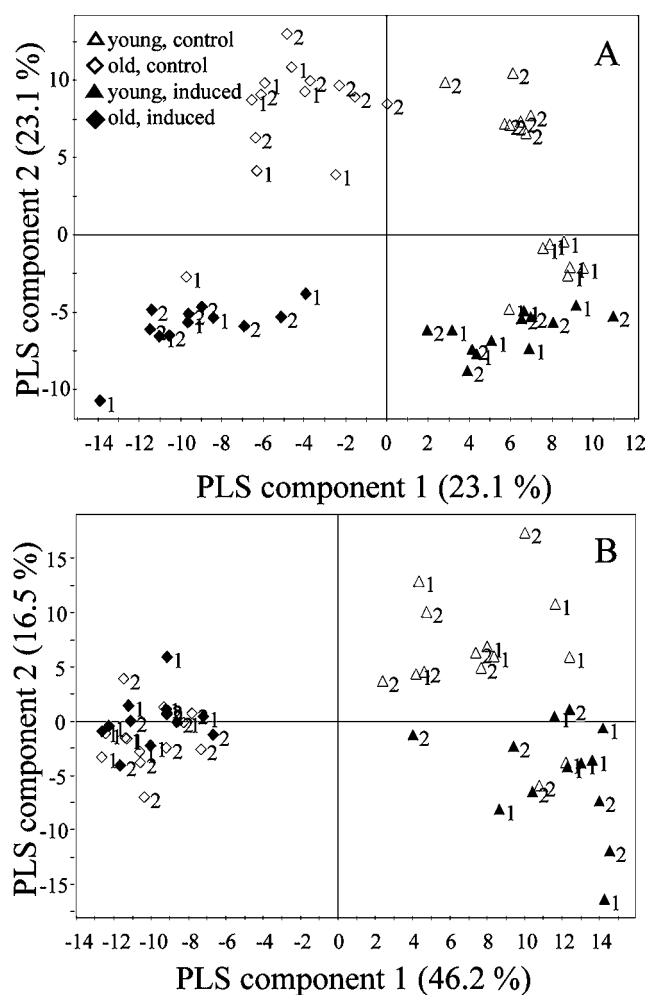

Figure 1. PLS-DA score plots showing groupings in ¹H NMR metabolomic profiles of non-GM ('1') and GM plants ('2'). Groupings occur between young and old leaves along component 1 and between healthy and (A) potato virus Y-infected or (B) aphid-infested leaves along component 2.
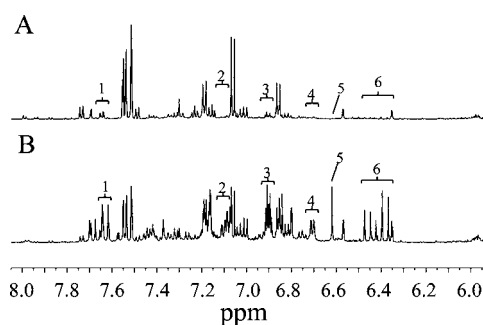

Figure 2. Differences in phenolic compounds in old leaves between (a) healthy and (b) potato virus Y-infected GM potato plants in the spectral region of 6.0−8.0 ppm. Some phenolics were increased in virus-infected plants (1 = unknown, 3 = unknown, 6 = 3-, 4-, and 5-caffeoylquinic acid), while others were synthesized de novo (2 = unknown, 5 = unknown).

experiments were spectral peaks assigned to the alkaloid trigonelline and phenolic compounds which were present in higher amounts in young leaves. Furthermore, sugars (glucose, sucrose) and choline were increased in young leaves. Levels of secondary metabolites are generally expected to be higher in,

Table 3. Sources of Variation in Relative α-Solanine and α-Chaconine Contents in Leaves in a Potato Virus Y (PVY) Infection Experiment
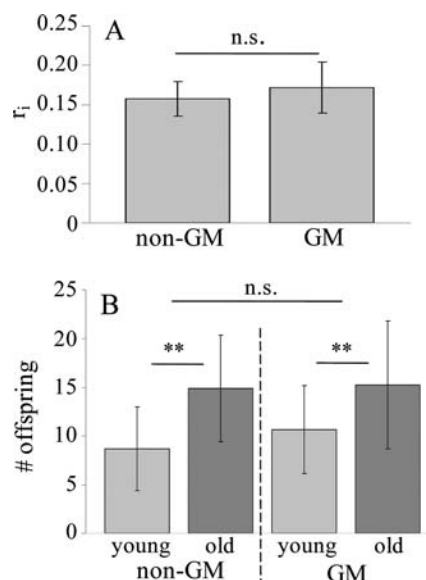
source of variation	df	SS	F	P
(a) α-solanine content				
leaf age	1	228.2	10.635	0.002
PVY infection	1	862.76	40.208	<0.001
residuals	49	1051.42		
(b) α-chaconine content				
leaf age	1	84.37	4.2854	0.044
PVY infection	1	639.52	32.4848	<0.001
leaf age: virus infection	1	102.47	5.205	0.027
residuals	48	944.96		

Table 4. Sources of Variation in Relative α-Solanine and α-Chaconine Contents in Leaves in an Aphid Herbivory (M. persicae) Induction Experiment

source of variation	df	SS	F	P
(a) α-solanine content				
leaf age	1	983.35	34.91	<0.001
residuals	50	1408.36		
(b) α-chaconine content				
leaf age	1	4826.00	106.01	<0.001
Residuals	50	2276.1		

with respect to fitness, more valuable plant parts such as young leaves, as part of an 'optimal defense' strategy.[27,28] Trigonelline is generally associated with biosynthesis regulation in response to abiotic stressors and with the accumulation of secondary metabolites.[29] The within-plant distribution of glycoalkaloids has previously been reported to show lower levels in the top leaves, to increase with leaf maturity, and to decrease again in older leaves.[30] A similar pattern was found in the plants of the aphid herbivory experiment: old leaves had lower glycoalkaloid contents than young leaves (Figure 3B, Table 4). Curiously, this relationship was reversed in the plants of the PVY infection experiment (Figure 3A, Table 3). Since these plants were 3 weeks younger, 'young and old' leaves in these plants may have been 'developing and mature' rather than 'mature and senescent' leaves, respectively.

The second largest effects on chemical profiles, following the effect of leaf age, were the effects of PVY infection and aphid herbivory (Tables 1 and 2). As apparent from the PLS-DA score plot of the PVY infection experiment (Figure 1A), control and PVY-infected plants are mostly separated along the second

Figure 4. (A) Instantaneous rates of population increase (r_i) of aphids *M. persicae* on 6-week-old GM and non-GM potato plants. (B) Number of offspring per individual aphid on excised leaves in Petri dishes. Error bars represent standard deviations.
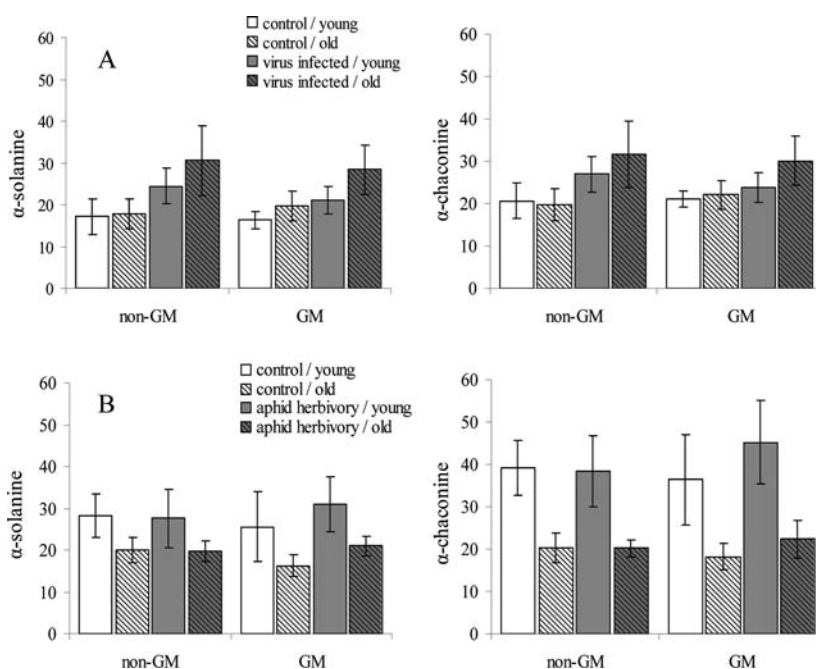
Figure 3. Relative amounts of the two main glycoalkaloids α-solanine and α-chaconine in a potato virus Y infection experiment (A) and an aphid herbivory experiment (B). Values are relative peak heights (square-root transformed) of ^{1}H NMR signals corresponding to H-6 of the aglycone. Error bars represent standard deviations.

component. Potato virus Y infection coincided with a general increase in phenolic compounds in the spectral region 6.0−8.0 ppm (Figure 2) including chlorogenic acid and its isomers. Sucrose and choline were reduced in infected plants. However, this shift in metabolomic profiles after PVY infection was not observed in young leaves of the non-GM cultivar where samples from healthy plants grouped together with samples from infected plants (Figure 1A). Both α-chaconine and α-solanine levels increased in response to PVY infection in both young and old leaves (Figure 3A, Table 3). Aphid herbivory had a weak effect on metabolomic profiles of old leaves but a stronger one in young leaves (Figure 1B). Leaves of aphid-induced plants had lower levels of sucrose and showed an increase of phenolics and malic acid. Glycoalkaloid levels were not affected by herbivory (Figure 3, Table 4).

Comparative Risk Assessment: Genetic Modification vs Chemical Baseline. In general, effects of genetic modification on chemical profiles were absent across infection treatments or leaf ages with one exception. A difference between GM and non-GM samples was only observed in young leaves of healthy plants. These young, healthy leaves of GM plants had lower levels of sugars and phenolic compounds compared to their non-GM counterparts. Glycoalkaloid levels were similar in both plant types across treatments (Figure 3, Tables 3 and 4). The observed difference was absent in older leaves of the same plants. It was also not found in PVY-infected plants or in any of the treatments in the aphid herbivory experiment. Thus, genetic modification affected metabolomic profiles only in a restricted developmental period (young leaves of 6-week-old plants) and under specific environmental conditions (healthy plants). Consequently, the genetic modification explained the least amount of variation in nonparametric MANOVA (Table 1) compared to the other treatments. In other words, when compared to the baseline of chemical variation, which in this study consisted of a combination of internal and external factors, the chemical changes caused by this genetic modification should be considered not biologically significant to plant−insect interactions.

An indication that the conclusion drawn from plant chemistry is indeed valid for plant−insect interactions may be the equal rate of population increase of aphids (M. persicae) on non-GM and GM plants during the aphid herbivory treatment (Figure 4A). We tested this more rigorously in a bioassay with parthenogenetic female aphids (M. persicae) on excised leaves of the two plant types using plants of the same age as the ones that were chemically profiled in the aphid herbivory induction experiment. The effect of leaf age was included in the bioassay as part of the baseline that had also been used to capture variation in chemical profiles. Again, aphid performance was not affected by genetic modification but was significantly lower on young leaves compared to old leaves (Figure 4B). Thus, while the effect with the largest influence on plant metabolomes did affect aphid performance, the minor effect of genetic modification did not. This suggests that the chemical baseline approach is valid at least for this specific plant−insect interaction. The pattern of aphid performance coincides with the relative amounts of glycoalkaloids that were found in plants of the same age in the herbivory induction experiment: we found higher amounts of glycoalkaloids in young leaves compared to old leaves and aphids performed less well on young leaves. While a causal relationship is not tested directly

here, the bioactivity of α-solanine and α-chaconine against aphids has been previously shown by refs 31 and 32.

We conclude that metabolomic studies can add important information to the assessment of ecological safety of genetically modified plants by revealing natural variation in plant chemistry as a relevant factor in plant−insect interactions. Selection of treatments that are included in a baseline is eventually a decision that has to be made by regulatory authorities. Once a set of criteria for a baseline is established, the comparative approach provides a workable framework for risk assessors.

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