Research Article

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Metabolomic fingerprint of cabbage resistance to *Mamestra brassicae* L. (Lepidoptera: Noctuidae)

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Abstract

BACKGROUND: Plants defend themselves from insect feeding by activating specific metabolic pathways. We performed a metabolomic analysis to compare the metabolome reorganization that occurs in the leaves of two genotypes of cabbage (one partially resistant and one susceptible) when attacked by *Mamestra brassicae* caterpillars.

RESULTS: The comparison of the metabolomic reorganization of both genotypes allowed us to identify 43 metabolites that are specifically associated with the insect feeding response in the resistant genotype. Of these, 19% are lipids or lipid-related compounds, most of which are modified fatty acids. These include glycosylated, glycerol-binding and oxidized fatty acids, the majority being associated with the oxylipin pathway. Some of the identified lipids are unlikely to be produced by plants and may be the result of biochemical reactions in the caterpillar oral secretions. A further 16% are phenylpropanoids. Interestingly, some phenylpropanoids were not present in the susceptible genotype, making them possible candidates for specific resistance-related compounds.

CONCLUSION: Our results suggest that glucosinolates do not have a clear role in the resistance to *M. brassicae* feeding on cabbage. Using an untargeted metabolomics approach, we associated the regulation of metabolic pathways related to lipid signalling and phenylpropanoid compounds with the resistance to this pest.

Keywords: cabbage; caterpillars; metabolomics; plant resistance

1 INTRODUCTION

Caterpillars are one of the major group of defoliators in nature. Adult lepidopteran insects generally deposit eggs on the underside of leaves and larvae start to feed on the leaf lamina after hatching. These larvae can defoliate a young plant in just a few days, which could be devastating for the plant, the crop and agricultural systems. One of the most devastating pests of vegetable crops throughout the world are the larvae of the cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae). These larvae are polyphagous and can feed on plants of more than 70 species.[1] However, it has been reported that plants from the Brassicaceae family are the most preferred for oviposition and feeding, especially the cabbage (*Brassica oleracea* var. *capitata*).[1,2] During the early stages of development, larvae feed on the outermost leaves, causing moderate damage to cabbage plants. From the fifth instar onwards, they show negative phototaxis and move into the crown of the plant.[3] Damage to the head and wrapper leaves of cabbages strongly reduces crop yield and marketability. In general, crop losses due to this pest may exceed 50%, especially under warm and humid conditions.[2,4]

Chemical insecticides are widely used to control larvae of *M. brassicae* in conventional farming. However, this is an undesirable means of control due to the impact that these treatments have on the environment, and human and animal health. Alternative methods for insect control are also important because many insect pests have developed resistance to conventional synthetic insecticides. Development of resistant cultivars is an effective way of controlling pests, but a deep knowledge of the resistance mechanisms is needed to effectively develop resistant cultivars. To date, studies have been focused on describing phenotypic mechanisms of resistance. Ploomi *et al.*[3] reported that resistance is associated with cabbage earliness. These authors concluded that, in general terms, early cultivars are less attractive to cabbage moth oviposition than late and mid-season cultivars. However, only six cultivars were used in the study, so more data are needed to confirm this hypothesis. Using a larger panel of cabbage cultivars, we identified genotypes that are partially resistant to caterpillars feeding under natural and artificial infestation conditions.[4] In a subsequent study, we established that a combination of antibiosis and antixenosis mechanisms are involved in resistance.[5]

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One differential characteristic of the Brassicaceae family is the production of specific defensive compounds called glucosinolates (GSLs). The role of these compounds in the resistance to lepidopteran pests has been extensively studied.[6–8] In their native form, GSLs are inactive against herbivores; however, upon tissue damage resulting from herbivore attack, GSLs are degraded to active molecules such as isothiocyanates (ITCs). Larvae of specialist insects are able to avoid the formation of these toxic derivatives.[9] However, some generalists, such as *M. brassicae*, can partially detoxify ITCs by conjugation to glutathione or amino acids;[10] therefore, the protective role of GSLs against these species may be limited.

In previous studies, we identified the local cultivar MBG-BRS0535 as a promising source of resistance to *M. brassicae*. This cultivar showed significantly higher resistance, compared to the other evaluated cultivars, to caterpillar feeding in field experiments and a significant antibiosis effect (high larval mortality rates) in a no-choice leaf feeding *in vitro* test, mainly at the prehead stage. The objective of the present study was to identify the metabolomic fingerprint of the resistant cultivar MBG-BRS0535 through a combination of targeted and untargeted metabolomics analysis.

2 MATERIALS AND METHODS

2.1 Plant material and experimental design

Two local cabbage varieties were evaluated in this study, based on their degree of resistance or susceptibility to *M. brassicae*. Variety MBG-BRS0409 was previously described as susceptible and MBG-BRS0535 as partially resistant to *M. brassicae* feeding.[4] Seeds were obtained from the Brassica germplasm bank at Misión Biológica de Galicia (MBG-CSIC) (Pontevedra, Spain). Sixty seeds per variety were sown in 2-L pots containing potting soil with peat, and plants were grown in a glasshouse for 4 weeks under controlled light (minimum 12 h per day) and temperature (10 °C at night, 25 °C during the day). Plants were watered twice a week.

Two months after sowing, 30 plants per genotype were infested with three second-instar *M. brassicae* larvae using a fine paintbrush. The leaves were covered with a nylon bag to prevent larvae dispersal. *Mamestra brassicae* eggs were supplied by the Institut National de la Recherche Agronomique (INRAE, Versailles, France). Neonates were reared under laboratory conditions using the method of Bucher and Bracken[11] for 3 days until the infestation date. Four days after infestation, all of the leaves of each plant were cut and pictures were taken in order to estimate the leaf damage. The damaged area of the leaves (cm^2) was measured using ImageJ.[12] For biochemical analysis, leaf samples were immediately collected in liquid nitrogen and conserved at −80 °C until extraction.

2.2 Glucosinolate analysis

The analysis of the GSL profile of the samples was carried out following previously described methodology,[13] with some modifications. First, 12 mg of freeze-dried cabbage leaf powder was mixed with 400 µL 70% (*v/v*) methanol preheated to 70 °C, 10 µL of lead acetate (PcAc) (0.3 mol L^{-1}), 120 µL ultra-pure water preheated to 70 °C and 20 µL of glucotropaeolin was added as an internal standard. The tubes were shaken in a microplate incubator (Model OVAN Orbital Midi, Badalona, Spain) at 250 rpm for 1 h and centrifuged at 3700 rpm for 12 min. Subsequently, 400 µL of the GSL extract was pipetted on an ion-exchange column with Sephadex DEAE-A25. Desulphation was carried out by addition of purified sulphatase (E.C. 3.1.6.1, type H-1 from *Helix pomatia*) (Sigma, St Louis, MO, USA) solution. Finally, the desulphated GSLs were diluted in 200 µL of ultra-pure water and 200 µL of 70% methanol, and kept at −20 °C for further analyses.

The chromatographic analyses were carried out using ultra-high-performance liquid chromatography (UHPLC Nexera LC-30 AD; Shimadzu, Kyoto, Japan) equipped with a Nexera SIL-30 AC injector and an SPDM20A ultraviolet (UV)-visible photodiode array detector. The UHPLC column was an X Select®HSS T3 [2.5 µm particle size, 2.1 mm × 100 mm inner diameter (i.d.)] from Waters (Milford, MA, USA) protected with a Van Guard pre-column. The oven temperature was set at 35 °C. GSLs were quantified at 229 nm and separated using the following method in aqueous acetonitrile, with a flow of 0.5 mL min^{-1}: 1.5 min at 100% water, an 11 min gradient from 5% to 25% (*v/v*) acetonitrile, 1.5 min at 25% (*v/v*) acetonitrile, a 1 min gradient from 25% to 0% (*v/v*) acetonitrile, and a final 3 min at 100% water. Specific GSLs were identified by comparing retention times (RTs) and UV spectra with standards.

2.3 Untargeted metabolomics

Freeze-dried powder (50 mg) was dissolved in 500 mL of 80% aqueous methanol and sonicated for 15 min. After centrifugation for 10 min (16 000 × *g*, at room temperature), the extract was filtered through a 0.20 µm micropore PTFE (polytetrafluoroethylene) membrane and placed in vials for further analysis. For metabolomic composition analysis, we used ultra-performance liquid chromatography (Thermo Dionex Ultimate 3000 LC) coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS/MS) (Bruker Compact™; Bruker, Karlsruhe, Germany) with a heated electrospray ionization (ESI) source. Chromatographic separation was performed on an Intensity Solo 2 C18 column (2.1 mm × 100 mm 1.7 µm pore size; Bruker Daltonics, Bremen, Germany) using a binary gradient solvent mode consisting of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The following gradient was used: 3% B (0–4 min), from 3 to 25% B (4–16 min), from 25 to 80% B (16–25 min), from 80 to 100% B (25–30 min), hold 100% B until 32 min, from 100 to 3% B (32–33 min), hold 3% B until 36 min. The injection volume was 5 µL, the flow rate was established at 0.4 mL min^{-1} and column temperature was controlled at 35 °C. MS analysis was operated in a spectra acquisition range from 50 to 1200 *m/z*. Both polarities (±) of ESI mode were used under the following specific conditions: gas flow 9 L min^{-1}, nebulizer pressure 38 psi, dry gas 9 L min^{-1}, and dry temperature 220 °C. Capillary and end plate offset were set to 4500 and 500 V, respectively. MS/MS analysis was performed based on the previously determined accurate mass and RT, and fragmented using different collision energy ramps to cover a range from 15 to 50 eV.

2.4 Statistical analysis

Analyses of variance (ANOVAs) for feeding resistance traits and GSL content were computed using the GLM procedure of SAS® software, version 9.4 (SAS Institute Inc., Cary, NC, USA, 2008). Comparisons of means were performed for each variety and trait using Fisher's protected least significant difference (LSD) at $P \leq 0.05$.

For untargeted metabolomics analysis, the algorithm T-Rex 3D from the MetaboScape 4.0 software (Bruker Daltonics) was used for peak alignment and detection. The generated dataset was imported into Metaboanalyst[14] for statistical analysis. In order to remove non-informative variables, data were filtered using the interquantile range filter (IQR). Moreover, Pareto variance scaling

was used to remove the offsets and adjust the importance of high- and low-abundance ions to an equal level. The resulting three-dimensional matrix (peak indices, samples and variables) was further subjected to statistical analysis. Features containing MS and MS/MS data from the resistant genotype were exported for global natural products (GNPs) feature-based molecular networking (FBMN) visible analyses. FBMN parameters were set as follows: mass tolerance for both precursor and product ions at 0.02 Da, minimum matched fragment ions at 4, and Top K at 10. Molecular networks were visualized with Cytoscape 3.9.1.[15]

To compare the performance of both genotypes, a partial least squares discriminant analysis (PLS-DA) was performed in order to identify the metabolic differences between the infested and control groups. PLS-DA models were cross-validated using quality assessment (Q^2) and R-squared (R^2) parameters. These statistics provide a quantitative measure of consistency between the predicted and original data, thus estimating the predictive ability of the model. A PLS-DA model is believed to be reliable when $Q^2 > 0.5$ and $R^2 > Q^2$. The PLS-DA model using the first principal component of variable importance in the projection (VIP) values was used to find differentially expressed metabolites. Based on VIP > 2, metabolites associated with resistance or pathogenicity or heat stress were distinguished. Features observed in the resistant genotype and not present in the susceptible genotype were selected for metabolite identification.

2.5 Tentative metabolite identification

Identification of putative metabolites was performed using accurate metabolite masses re-ported in different publicly available databases, such as METLIN (https://metlin.scripps.edu), KEGG (https://www.genome.jp), Pubchem (https://pubchem.ncbi.nlm.nih.gov), HMDB (https://hmdb.ca) and Plant Metabolic Network (https://plantcyc.org). All databases were accessed between 1 August 2021 and 6 June 2022. Additionally, further partial identification of the most significant metabolites was performed by comparison of MS/MS fragmentation patterns against reference compounds found in the earlier-mentioned databases and the literature.

3 RESULTS

Prior to performing any metabolomic analysis, we confirmed the resistance of the cultivar MBG-BRS0535 in our experimental conditions. This cultivar showed a significantly lower area damaged by caterpillar feeding and a lower percentage of the leaf damaged than the susceptible cultivar (MBG-BRS0409) in the pre-heading developmental stage (Fig. 1).

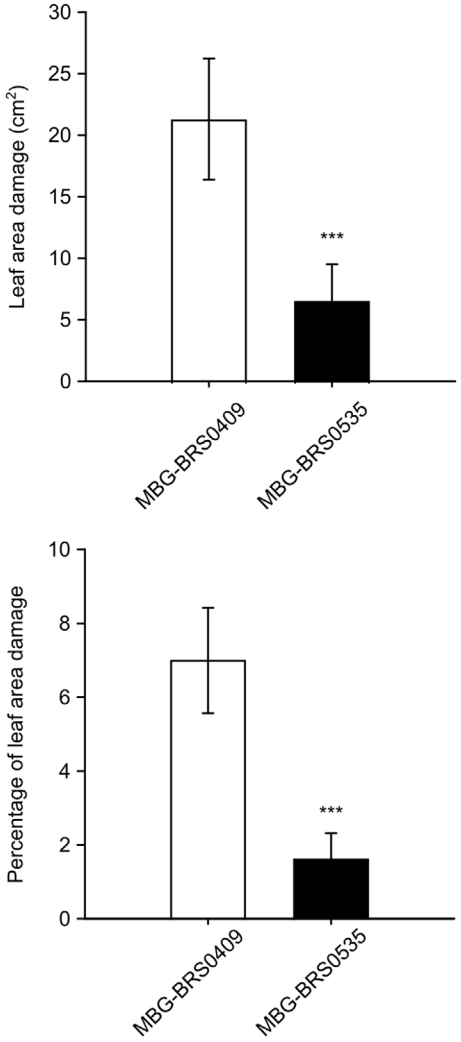

Figure 1. Means of the damaged leaf area and percentage of leaf damage by *Mamestra brassicae* in two genotypes of cabbage; MBG-BRS0409, susceptible genotype; MBG-BRS0535, partially resistant genotype. Error bars denote ± standard error. $n = 15$.

3.1 Role of glucosinolates in resistance to caterpillar feeding

Both genotypes have similar constitutive levels of total GSLs content. The level of GSLs increases in both genotypes after insect feeding, although this increase is not significant in the resistant genotype (Fig. 2(A)). Chemically, GSLs are divided into two major groups, indolic and aliphatic GSLs. In control conditions, both genotypes have equivalent levels of total aliphatic and indolic GSLs (Fig. 2 (B,C)). The level of aliphatic GSLs decreases after insect feeding in both genotypes, although this reduction is only significant in the susceptible genotype. We detected and quantified the levels of two aliphatics: glucoiberin (GIB) and sinigrin (SIN). The major effect of feeding is seen in the SIN content, which decreases in a similar way in both genotypes, although again this reduction is only significant in the susceptible genotype. In contrast, the levels of indolic GSLs increase after feeding, and this increase is significant in both genotypes. This increase in indolic GSLs is predominantly due to the accumulation of glucobrassicin (GBS) after caterpillar feeding, which is significant in both genotypes. The accumulation of neoglucobrassicin (NeoGBS) in the susceptible genotype after insect feeding is also notable, whereas in the resistant genotype levels remain similar to those observed in control conditions.

Thus, our results indicate that cabbage plants accumulate indolic GSLs after *M. brassicae* feeding, but that this increase is not related to resistance since both the resistant and susceptible genotypes show a similar increase in indolic GSLs. However, both genotypes tend to show a decrease in the levels of aliphatic GSLs after caterpillar feeding. In conclusion, we cannot establish a clear association between the levels or type of GSLs and resistance to *M. brassicae* feeding.

3.2 Untargeted metabolomic profiling

In order to identify the group of compounds that may be associated with the resistance to caterpillar feeding, we performed an untargeted metabolomic analysis. We compared the

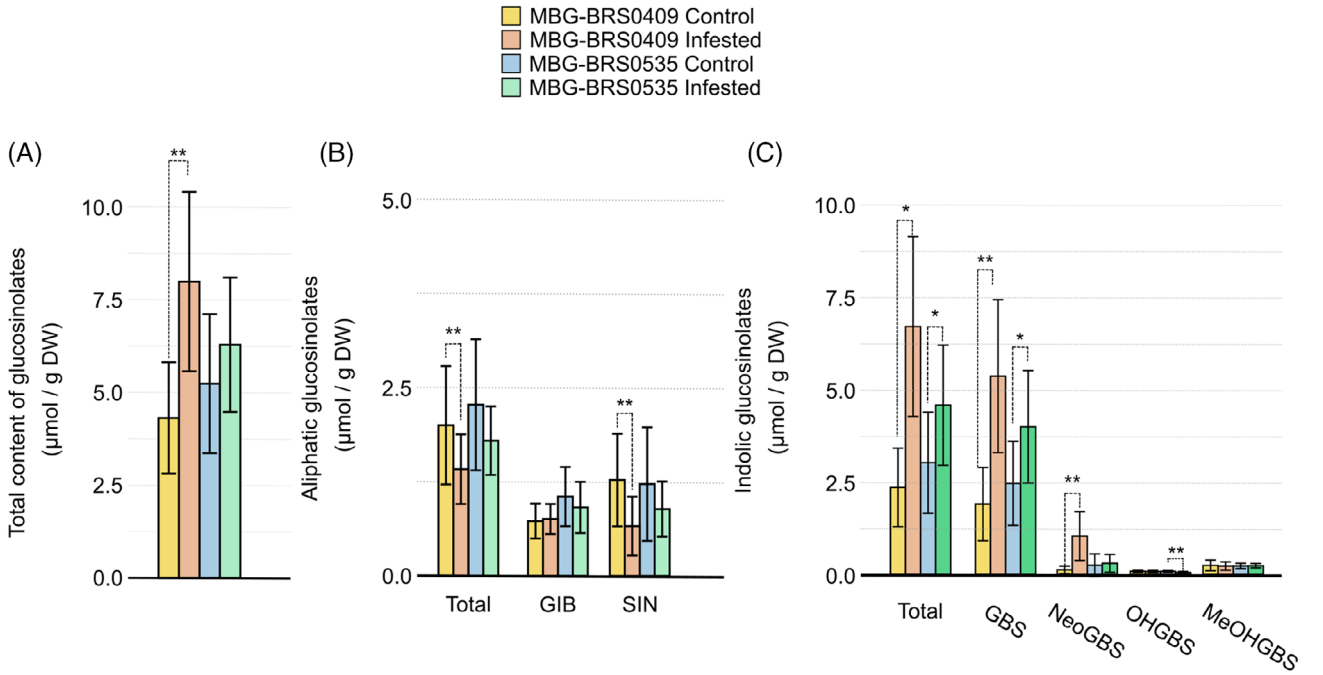

Figure 2. Means of total glucosinolate content (A), aliphatic (B) and indolic glucosinolates (C), in two genotypes of cabbage (MBG-BRS0409, susceptible genotype; MBG-BRS0535, partially resistant genotype) infested with larvae of *Mamestra brassicae*. Error bars denote ± standard error. $n = 15$. Bars labelled with different letters indicate significantly different results ($P < 0.05$; one-way ANOVA).

metabolomic profile of infested and control plants of each genotype separately. Values of $Q^2 > 0.8$ and $R^2 > 0.9$, and $Q^2 > 0.6$ and $R^2 > 0.9$ were obtained in the analysis of the susceptible and partially resistant genotypes, respectively. These results indicate a good predictive power of the models. Overall, 128 features were identified to be differentially accumulated in the resistant genotype. The FBMN analysis of these features allowed us to identify molecular groups that are over-represented in the resistant genotype (Fig. 3). Based on the MS2 fragmentation pattern, most of these features are classified into two groups: phenolic compounds (further classified into two classes of phenolics: flavonoid glycosides, and hydroxycinnamic acids and derivates) and benzenesulfonamides. Three groups can be classed as compounds associated with lipid metabolism: fatty amides, glycerophosphocholines and glycosylglicerols. Finally, the carbohydrates and conjugates group includes several GSLs and related compounds.

In order to select the most promising candidates that may be responsible for the observed resistance, we selected only those metabolites with a VIP score > 2 in the resistant genotype exclusively. This comparison yielded 69 features, which we used for further identification. We manually filtered this database, and features with an intensity correlation coefficient > 0.7 among samples and a $\Delta RT < 3$ s were carefully studied in order to remove those that were most likely due to in-source fragmentation of real metabolites.

After this analysis, we finally obtained a database of 43 metabolites (22 identified in positive ionization mode and 21 in negative ionization mode) (Fig. 4). These metabolites were tentatively identified based on exact mass and MS/MS fragmentation pattern. The analysis of this database indicates that, in spite of being a significant group when we compare the response of inoculated *versus* control plants of the resistant genotype, benzenesulfonamides also respond in the susceptible genotype. A total of 19% of the specific metabolites are lipid or lipid-related compounds, most of which are modified fatty acids, including glycosylate, glycerol-binding and oxidized fatty acids. Another 16% are phenolic compounds. Among these compounds, we found a significant number of hydroxycinnamic acids, most them being derivatives of ferulic acid. Three GSLs (the aromatic gluconasturtiin and the indolics glucobrassicin and methoxyglucobrassicin) show a specific response in the resistant genotype. Our data also confirm that identification of metabolites is a bottleneck in plant metabolomics analysis, since 42% of the metabolites we identified in our analysis could not be assigned to any known molecule.

4 DISCUSSION

Plants defend themselves from defoliators by activating an intricate network of signalling that results in deep metabolomic reorganization.[16] The understanding of such mechanisms is necessary to design the best breeding strategies to develop resistant genotypes. In a recent article, we have identified a cabbage genotype resistant to *M. brassicae* caterpillar feeding.[4]

Our current metabolomic analysis reveals that there are two major groups of compounds associated with resistance to *M. brassicae* feeding in cabbage: lipid and lipid-like molecules and phenylpropanoids. Lipids and lipid derivatives have been extensively associated with plant defences to both pathogens and herbivores.[16–19] The central core of lipid signalling in plants is the octadecanoid pathway, which leads to the biosynthesis of oxylipins. Oxylipins are synthesized from unsaturated octadecanoid acid, although in some plant species, such as *Arabidopsis thaliana*, jasmonates can also be synthesized from hexadecatrienoic acids.[20] We identified two fatty acids, hexadecatrienoic acid and linolenic acid, as important metabolites associated with resistance in our metabolomic model. The source of these fatty acids is membrane lipids, likely from monogalactosyldiacylglycerol (MGDG).[21] We identified a monogalactosylmonoacylglicerol

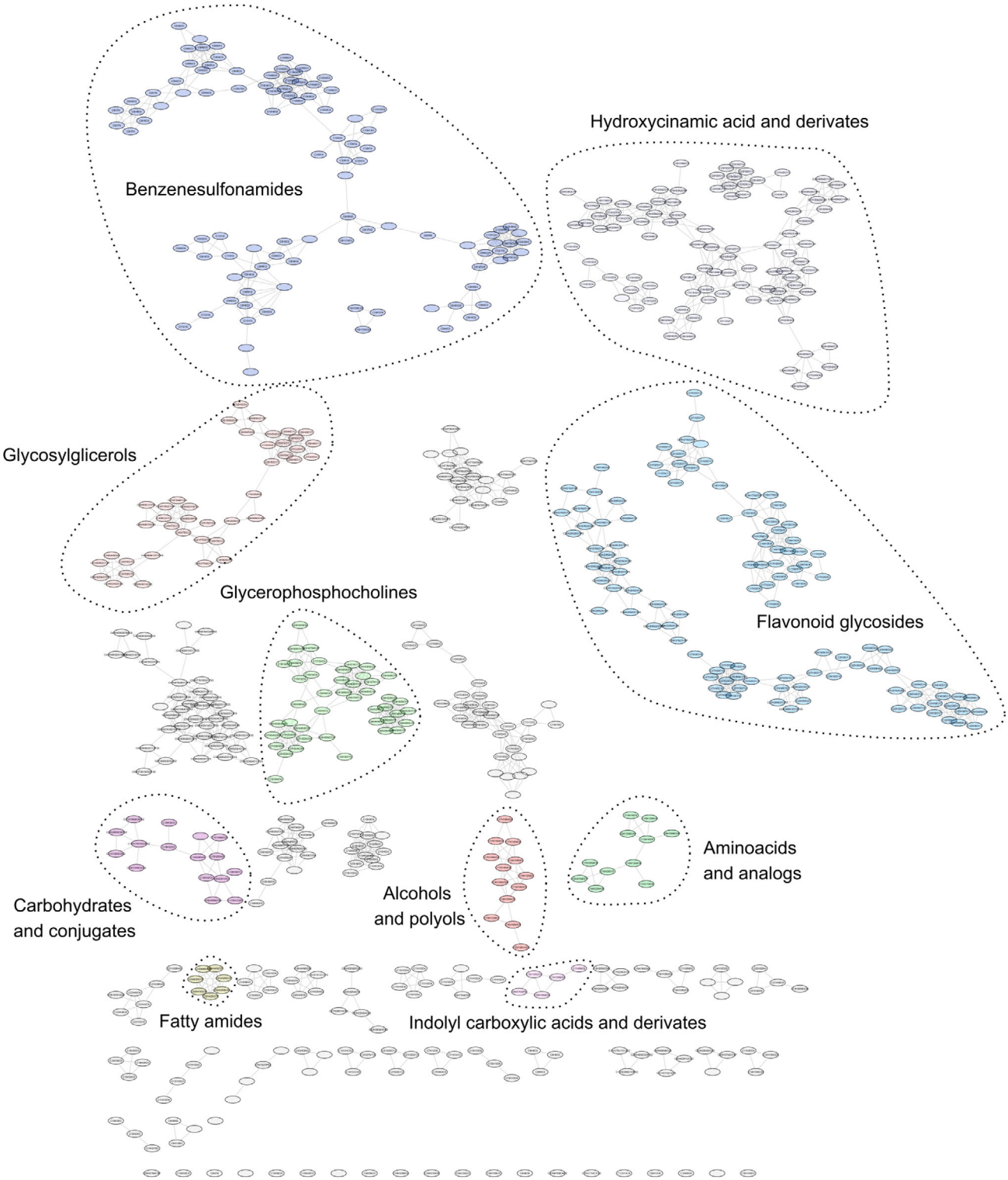

Figure 3. Graphical representation of the feature-based molecular networking (FBMN) analysis performed in GNPs (global natural products social molecular networking) on a resistant genotype (MBG-BRS0535) to *Mamestra brassicae* feeding. Metabolites are grouped based on hierarchical chemical classification performed using Classyfire.

(MGMG) with the acyl group consisting of a linolenic acid, which could be consistent with the release of linolenic acid from membranes. Our analysis also indicates a metabolite that could be identified as a phospholipid (801.47 *m/z*). Phospholipids are the second major lipid constituents of plant membranes. Although we could not assign an unequivocal molecular formula to this metabolite, the MS/MS fragmentation pattern shows the presence of a predominant 184.07 *m/z* fragment, which is characteristic of the presence of a phosphocholine headgroup and fragments at 261.21 and 247.16 *m/z* associated with the presence of a linolenic acid.

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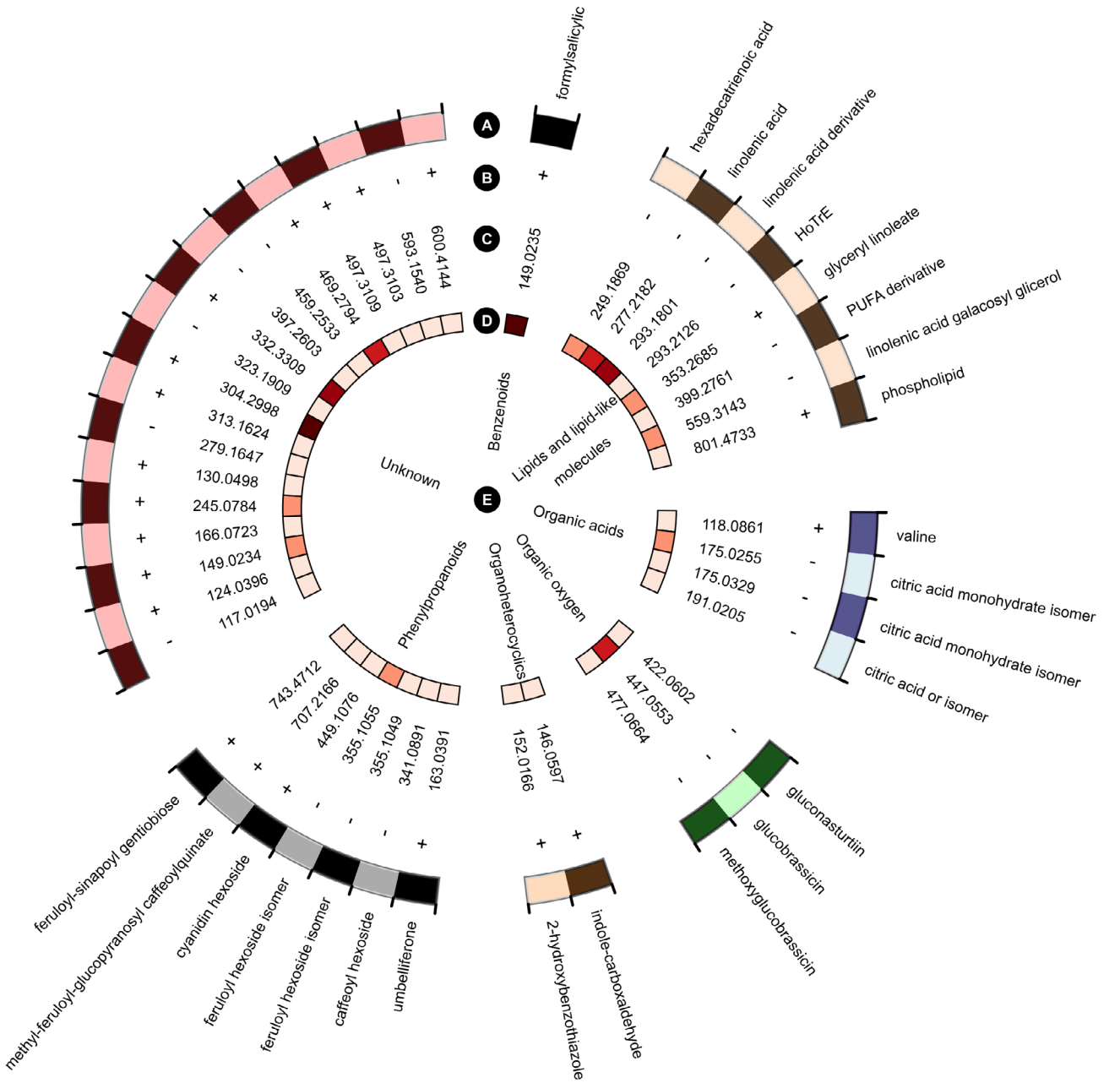

Figure 4. Circular representation of metabolites with a VIP score > 2 in a supervised PLS-DA of untargeted metabolomic data. (A) Tentative name assigned to selected masses; (B) ionization mode; (C) exact mass of selected metabolites; (D) heatmap representation of VIP score values (darker colours indicate higher VIP scores); (E) aggrupation of compound superclasses, assigned based on hierarchical chemical classification performed using Classyfire.

We also identified several metabolites that can be classified as derivatives of polyunsaturated fatty acids (mainly linolenic acid). Based on the literature, we identified one of these metabolites as hydroxy linolenic acid (HOT; $C_{18}H_{30}O_3$).[22] Hydroxylated derivatives of linolenic acid are produced from hydroperoxide (HPOT) from the activity of lipoxygenases or dioxygenases and a subsequent reduction due to peroxidase activity.[23] However, to the best of our knowledge, only 9-HPOT or 13-HPOT products of lipoxygenase activity and 2-HPOT from dioxygenase activity have been described in plants,[24,25] whereas the fragmentation pattern of the HOT molecule that we identified in our analysis is consistent with hydroxylation at carbon 17 or 18 (Fig. 5). Interestingly, a 17-hydroxylated form of linolenic acid has been described as part of the volicitin molecule, which is a lipid-derived compound identified from the oral secretions of beet armyworm caterpillars.[26] Volicitin acts as elicitor of plant defences and volatile production. Whether the hydroxylated form of linolenic acid we identified in our analysis is produced by *M. brassicae* caterpillars requires further investigation.

The second major group of compounds that we identified as important in cabbage resistance to *M. brassicae* are phenylpropanoids. These metabolites are major components of plant metabolism. They are biosynthesized through the shikimate pathway, which links carbohydrate metabolism with the biosynthesis of aromatic compounds.[27] Phenylpropanoids are phenolic compounds derived from phenylalanine. We identified seven

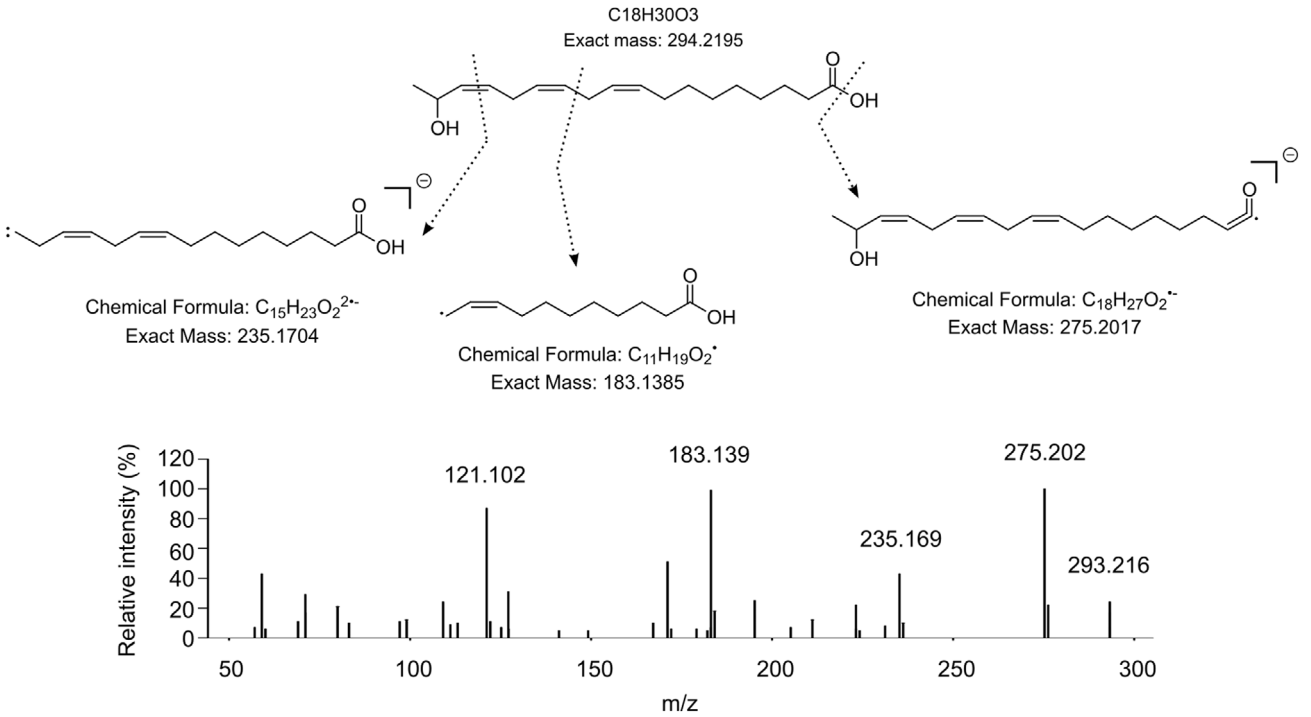

Figure 5. Mass spectrum of putative hydroxy linolenic acid (*m/z* 294.21). Fragments fit with the theoretical fragmentation pattern of a 17-hydroxy linolenic acid.

phenylpropanoids that can be classified as hydroxycinnamic acids (esters of ferulic, caffeic and sinapic acids, and conjugates), flavonoids (cyanidin hexoside) and coumarins (umbelliferone). The different groups of phenylpropanoids are mainly associated with various plant responses to herbivores. For instance, hydroxinamic acids have been associated with cell-wall reinforcement by cross-linking hemicellulose,[28] whereas flavonols and coumarins act as deterrent compounds.[16,29] Interestingly, we could not identify three of these metabolites in the susceptible genotype: cyanidin hexoside, methyl-feruloyl-glucopyranosyl caffeoylquinate and feruloyl-sinapoyl gentiobiose. This indicates that these compounds may be especially relevant in cabbage resistance to herbivores.

A special mention is required for the results we obtained from the analysis of GSLs. The targeted analysis suggests that GSLs have very little, if any, impact on cabbage resistance. Despite being a generalist herbivore, larvae of *M. brassicae* are able to partially inactivate ITCs, biologically active products of GSLs, by conjugation to glutathione and cysteine.[10] The toxic effect of GSLs on this species has been demonstrated, since larvae of *M. brassicae* grow more quickly when consuming plants of *Arabidopsis* mutants impaired in the synthesis of these compounds.[7,30] However, this effect does not seem to be as clear in cultivated species. Poelman *et al.*[31] reported no correlation between the performance of *M. brassicae* larvae and the content of GSLs in eight cultivars of white cabbage (*Brassica oleracea* var. *alba*). Santolamazza-Carbone *et al.*[32] reported a dose-dependent effect of GSLs. Larvae fed on leaves from juvenile kale plants (*Brassica olearece* var. *acephala*) with lower GSL content do not show differences in development between genotypes with high and low GSL concentrations, whereas significant differences were observed in adult plants of the same genotypes. In a preliminary analysis, we did not observe any correlation between the GSL content and resistance to herbivory in 16 local cabbage varieties.[4,33] It has been postulated that the performance of lepidopterans could be affected in cultivated plants due to the enhanced nutrient levels due to artificial selection, which may compensate for the negative effect of GSL consumption.[31]

In our analysis, there was also an apparent contradiction between results obtained in the targeted and untargeted metabolomic analysed, since three GSLs appear as important metabolites in the untargeted analysis. This result may be explained by the statistical analyses used in these approaches. Saccenti *et al.*[34] reported different reasons why a univariate analysis could give different results than those observed in a multivariate analysis. The more straightforward explanation in this case is that metabolite abundances may complement each other in the prediction of the separation between the susceptible and resistant genotypes. These interactions are then only considered when the response is modelled in a multivariate analysis.

In conclusion, the partial resistance to *M. brassicae* observed in the cabbage genotype MBG-BRS0535 is due to a reorganization of the metabolome upon insect feeding. This reorganization involves two major groups of defensive compounds, lipids and lipid-like molecules, and phenolic compounds. We also identified a new putative hydroxylated linolenic acid that could act as an elicitor of the plant defensive response.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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