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A Fast and Precise Method To Identify Indolic Glucosinolates and Camalexin in Plants by Combining Mass Spectrometric and Biological Information

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Supporting Information

ABSTRACT: In this manuscript, a fast and accurate identification and quantitation by mass spectrometry of indolic glucosinolates and camalexin involved in defense in *Arabidopsis thaliana* are described. Two elicitation systems, inoculation with *Botrytis cinerea* and treatment with $AgNO_3$, were used in Col-0 wild-type and mutant genotypes impaired in the biosynthesis of the selected metabolites. Identification of analytes was carried out by nontargeted LC/ESI-QTOF-MS profiling. Confirmation of indolic glucosinolates and camalexin was achieved by their absence in the *cyp79B2/B3* and *pad3* mutants as well as their respective fragmentation upon collision-induced dissociation. Camalexin accumulation was induced only after $AgNO_3$ treatment, whereas all indolic glucosinolates were constitutively present. Inoculation with *Botrytis* did not influence camalexin concentration but caused most aliphatic and indolic glucosinolates contents to decrease. Only the *pen 3.1* mutant showed increased indolic glucosinolate levels after *Botrytis* or $AgNO_3$ treatments. In addition, profiles of secondary metabolite in nontreated Col-0 and mutant plants were analyzed by means of partial least squares coupled to discriminant analysis (PLS-DA), and differences in the basal levels of indolic glucosinolates and tryptophan between *cyp79B2/B3* plants and the rest of genotypes, including Col-0, were found. This probably has to be taken into consideration when comparing stress responses of Col-0 and *cyp79B2/B3*. The use of mutants carrying alterations in biosynthetic pathways is proposed as a useful strategy to identify secondary metabolites.

KEYWORDS: *metabolomics, LC/ESI-QTOF-MS, abiotic stress, biotic stress, secondary metabolism, phytoalexins*

INTRODUCTION

In response to environmental stress conditions, plants activate a plethora of responses including changes in growth and developmental patterns and primarily severe alterations in metabolic processes such as glycolysis, tricarboxylic acid cycle, and aminoacid biosynthesis.[1,2] Abiotic stress conditions, such as drought or salinity, directly affect carbon assimilation and consequently alter plant primary metabolism.[2,3] It has been shown that the stress conditions also affect the accumulation of different minor compounds with a less clear interconnection with photosynthesis.[4,5] These compounds, collectively referred to as secondary metabolites, are of very diverse origins, and their composition and biosynthesis are not yet well understood. Their biological roles are also very diverse and include antioxidant properties, defense, or signaling.[6−8] Interestingly, they are specific for given species or botanical families. Therefore, these compounds could be used as markers to certify plant material from different origins,[9] an important aspect when dealing with many species of importance to human nutrition and health.

Among all edible plants, Brassicaceae is the botanical family including more species important in agriculture and human nutrition, for example, cabbage, turnip, rapeseed, etc. Glucosinolates, the most abundant compounds of this family, are sulfur- and nitrogen-containing molecules that carry a hydroxyaminosulfate group and β-thioglucosyl residue attached to a variable side chain.[10]

Glucosinolates are synthesized from aminoacids by conversion to the respective acetaldoxime derivative by cytochromes (CYP79F1/F2 and CYP79B2/B3, Figure 1, adapted from ref 11). Depending on the side chain, glucosinolates can be divided into aliphatic (derived from alanine, methionine, valine, or leucine), aromatic (derived from tyrosine or phenylalanine), and indolic (which are mainly derived from tryptophan).[11,12] These compounds have been primarily associated with defense against biotic stressors such as herbivores, fungi, and also bacteria acting as growth deterrents or as powerful toxic substances. To exert their biological activity, the β-thioglucosyl residue needs to be cleaved by a specific enzyme known as myrosinase, a type of thioglycosidase. This enzyme cleaves the β-thioglycosidic bond, yielding mainly isothiocyanates, thiocyanates, and nitriles, which are responsible for the biological activity of glucosinolates.[12,13]

Among all known glucosinolates (more than 120 structures characterized so far), the indolics are the most important involved in stress defense.[12−14] In the model plant species, *Arabidopsis thaliana*, these metabolites are derived from tryptophan after cleavage with a CYP79 enzyme that converts the precursor tryptophan to indole-3-acetaldoxime. This metabolite is the primary precursor of both indolic

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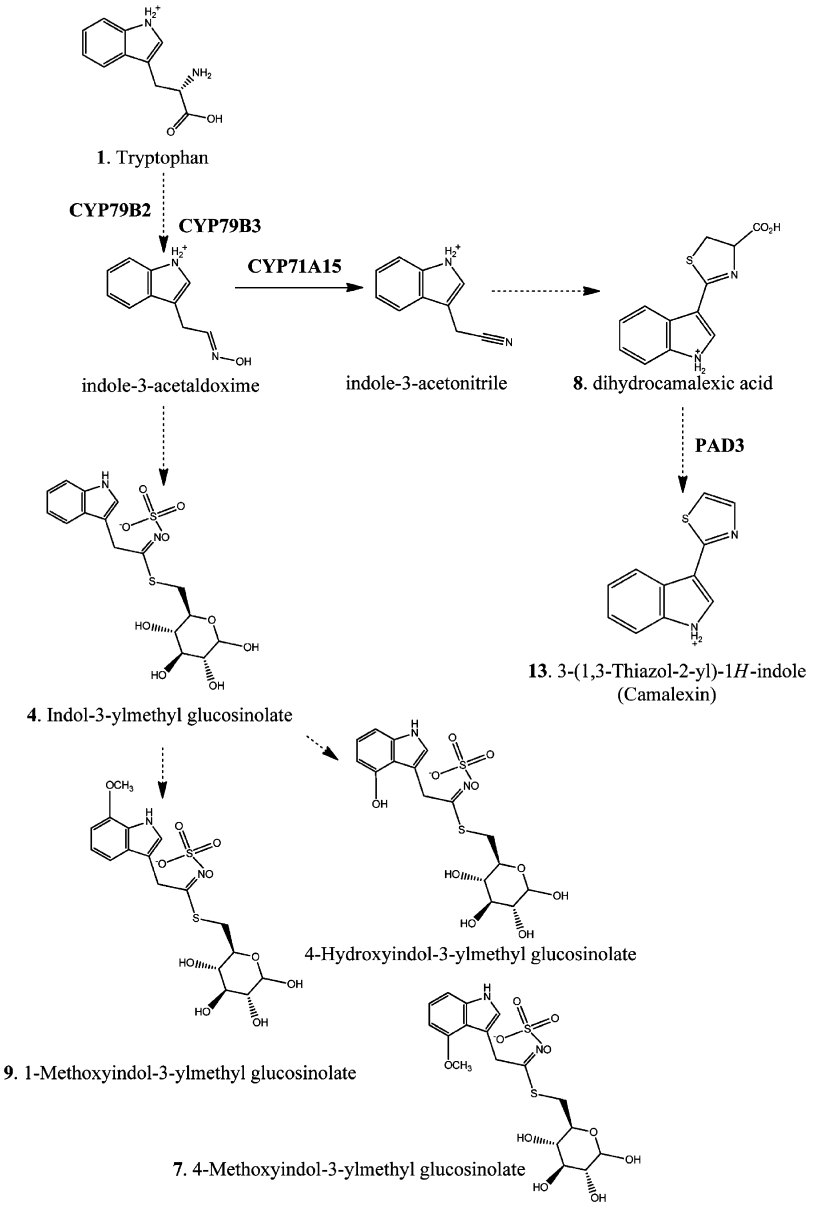

Figure 1. Metabolic pathway of indolic glucosinolates with names of key biosynthetic steps highlighted. The compounds detected in the profiles are indicated in Arabic numerals. The scheme was adapted from ref 11.

glucosinolates and camalexin [3-(1,3-thiazol-2-yl)-1*H*-indole], the main phytoalexin. Indolic glucosinolates are always present in plant tissues, although their relative levels might change depending on the specific genetic background, tissue or developmental stage,[14] and in response to environmental cues.[14,15] On the contrary, phytoalexins are only produced under certain stress conditions, such as pathogen elicitation, heavy metal toxicity, etc.[6] In this sense, it is likely that the production of glucosinolates in *A. thaliana* under certain stress conditions is tightly regulated.[16,17]

Stress responses might reflect whole plant performance, physiological status, or even a genetic modification. Therefore, there is an increasing demand for high-throughput methods to evaluate slight variations in minor compounds.[4,9] In this sense, LC/MS-based metabolite profiling techniques provide an unbiased methodology for the analysis of semipolar compounds.[9,18] Current metabolite profiling platforms take advantage of modern mass spectrometers (as well as other powerful techniques, such as NMR) to gather molecular information on metabolites to aid in structural elucidation and identification. However, mass spectrometry is not enough to identify all metabolites present in a given sample, and it becomes necessary to coinject reference standards, which are not always commercially available. In addition, in the analysis of intact glucosinolates, it is crucial to first suppress all myrosinase activity by boiling the samples to prevent cleavage to take place.[19]

In metabolite profiling techniques, a bottleneck is the identification of metabolites. In high-resolution mass spectrometry, such as hybrid quadrupole time-of-flight (QTOF), identification of metabolites starts by formulating a hypothesis on the identity of a metabolite based on the ion composition of the mass chromatographic feature, search of informative fragmentations, and calculation of elemental composition. However, the unbiased identification of a certain metabolite is limited to the commercial availability of analytical stand-

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ards.[18] This is of special relevance in the case of indolic glucosinolates, for which no commercial standards are available.[19] To overcome this problem, a set of *Arabidopsis* mutants lacking different key enzymes in indolic glucosinolate or camalexin biosynthesis were included in this study. In addition, two different adverse conditions were assayed: biotic stress by inoculating plants with *Botrytis cinerea* conidia, a necrotrophic fungal plant pathogen to which *A. thaliana* has developed a nonhost resistance,[20] and abiotic stress by spraying plants with a $AgNO_3$ solution, which induces the accumulation of camalexin.[20,21]

In this work, the main objective was to unequivocally identify indolic glucosinolates and camalexin in *Arabidopsis* plants by using the biological information obtained from mutants impaired in the biosynthesis of these compounds. In addition, the impact of biotic and abiotic stress on glucosinolate and camalexin accumulation and the difference in metabolite composition among *Arabidopsis* mutants and wild-type plants were analyzed.

MATERIALS AND METHODS

Reagents and Standards. Liquid chromatography−mass spectrometry (LC-MS) grade acetonitrile from Panreac (Barcelona, Spain) and Milli-Q water (Millipore Corp., Billerica, MA) were used for the liquid chromatography/electrospray ionization−quadrupole time-of-flight mass spectrometry (LC/ESI-QTOF-MS) analyses. Formic acid (Panreac) was used as the mobile phase modifier. For extractions, methanol (MeOH LC-MS grade) from Panreac was used. Biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) and leucine-enkephalin were obtained from Sigma-Aldrich (Madrid, Spain). For stress treatments and *Botrytis* culture, $AgNO_3$ and KH_2PO_4 were purchased from Panreac.

Plant Cultivation and Stress Treatments. Plant material used in the experiments was *A. thaliana* accessions Col-0 as wild type and mutants *pad3*,[22,23] *cyp79B2/B3*,[24] and *pen3.1*,[20] all in the Col-0 background. Seeds were germinated in jiffy pellets (Jiffy Products España S.L.U., Murcia, Spain) and allowed to grow for 2 weeks in a growth chamber with 8 h of illumination at 21 °C and 16 h of darkness at 18 °C. After that time, seedlings were transplanted to individual jiffy pellets and allowed to grow for 6 more weeks before imposition of treatments. For abiotic and biotic stress treatments, plants were sprayed with a 5 mM $AgNO_3$ solution or with a *B. cinerea* conidia suspension (5×10^4 conidia/mL in Gamborg medium, supplemented with 10 μmol/L sucrose and KH_2PO_4, as described in ref 25). Control plants were sprayed with tap water. After 24 h of each treatment, leaf rosettes of the different genotypes were harvested, immediately frozen in liquid nitrogen, ground to fine powder, and stored at −80 °C until analysis. Two independent biological replicate experiments were performed, and three independent sample replicates per sample group and experiment were analyzed by LC/ESI-QTOF-MS.

Extraction. Extraction was performed essentially as previously described in ref 19 with slight modifications. Briefly, 500 μL of 70% methanol supplemented with biochanin A at 1 mg/L (internal standard, IS) was added to 0.1 g of frozen leaf powder. After 10 min of sonication, samples were incubated for 15 min at 80 °C in a water bath to stop myrosinase activity. Extracts were allowed to cool down at room temperature and centrifuged at 10000*g* for 10 min at 4 °C. Prior to UPLC-QTOF-MS analysis, supernatants were filtered through 0.2 μm PTFE syringe filters (Whatman International Inc., Kent, United Kingdom).

Instrumentation and Conditions. Chromatographic separations were performed on an Acquity SDS system (Waters Corp. Ltd., Milford, MA) interfaced to a QTOF Premier from Micromass Ltd. through an ESI source. Two reversed-phase columns were evaluated as follows: 100 mm × 2.1 mm i.d., 5 μm, XTerra C18 LC-MS (Waters), and 100 mm × 2.1 mm i.d., 2.1 μm, ProntoSIL C18SH (Bischoff Chromatography, Leonberg, Germany). Samples were injected in the UPLC system in 10 μL aliquots using the partial loop-filling option. Separations were carried out using two gradients at a flow rate of 300 μL/min. Conditions of gradient 1 were as follows: 0−2 min, isocratic 95% A [water:formic acid, 99.9:0.1 (v/v)] and 5% B [acetonitrile:-formic acid, 99.9:0.1 (v/v)]; 2−27 min, gradient 5−95% B; 27−30 min, return to initial conditions; 30−35 min, re-equilibration period. Conditions of gradient 2 were as follows: 0−2 min, isocratic 5% B; 2−17 min, gradient 5−95% B; 17−20 min, return to initial conditions; 20−25 min, re-equilibration period. During analyses, the column temperature was maintained at 40 °C, and samples were maintained at 5 °C to slow down degradation.

Samples were analyzed in both negative and positive ionization modes. Two functions were set in the instrument: in function 1, data were acquired in profile mode from 50 to 1000 Da using a scan time of 0.2 s and a collision energy of 2 eV; in function 2, the scan range was the same, but a collision ramp between 4 and 65 eV was set. During all measurements, the electrospray capillary voltage was set to 4 kV, and the cone voltage was set to 25 V. The source temperature was maintained at 120 °C, and the desolvation gas temperature was set at 350 °C. Argon was used as the collision gas, and nitrogen was used as the nebulizer as well as desolvation gas set at 60 and 800 L/h, respectively. Exact mass measurements were provided by monitoring the reference compound lockmass leucine-enkephalin.

Data Processing. Data were processed using Masslynx v.4.1. Raw data files were converted to netCDF format using the application *databridge* from Masslynx and processed using the xcms package.[26] Chromatographic peak detection was performed using the matchedFilter algorithm,[9] applying the following parameter settings: snr = 3, fwhm = 15 s, step = 0.01 D, mzdiff = 0.1 D, and profmethod = bin. Retention time correction was achieved in three iterations applying the parameter settings minfrac = 1, bw = 30 s, mzwid = 0.05 D, span = 1, and missing = extra = 1 for the first iteration; minfrac = 1, bw = 10 s, mzwid = 0.05 D, span = 0.6, and missing = extra = 0 for the second iteration; and minfrac = 1, bw = 5 s, mzwid = 0.05 D, span = 0.5, and missing = extra = 0 for the third iteration. After final peak grouping (minfrac = 1, bw = 5 s) and filling in of missing features using the fillPeaks routine of the xcms package, a data matrix consisting on feature × sample was obtained. In these data sets, only consistent mass signals were considered, whose significance level of *P* values (*t* test, two-tailed, unequal variances) was lower than 0.05.

Data mean comparisons were performed with Statgraphics Plus V.5.1. software (Statistical Graphics Corp., Herndon, VA). One-way analysis of variance (ANOVA) was performed to assess differences between treatments and genotypes considering a significance value of 0.05. Posthoc data mean comparisons were achieved with a least significant difference (LSD) test. For multivariate analysis of the whole data set, peak detection and retention time correction of control samples of each genotype were performed using a similar set of parameters as described above. After filling in missing chromatographic mass features and removal of inconsistent features, principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were performed using Simca-P (v 11.0) software (Umetrics, Umea, Sweden).

RESULTS AND DISCUSSION

Optimization of Chromatography. Until now, few studies have described the use of UPLC-MS for the analysis of glucosinolates and camalexin. Recently, a new powerful identification tool has become available: the hybrid QTOF mass spectrometer. In addition to the improved characteristics of TOF instruments, they offer the possibility of performing MS/MS acquisitions to obtain product ion spectra with accurate mass, which is sometimes necessary to aid in the identification of compounds or even differentiate between structural isomers.[22,27]

In this first part of this work, the objective was to develop a nontargeted metabolite profiling methodology for the analysis of variations in indolic glucosinolate and camalexin levels in *A.*

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Table 1. List of Metabolites Identified in *Arabidopsis* Leaf Extracts[a]

no.	compd name	formula	quantifier ion type, m/z	R_t (min)	pseudomolecular ion			CID fragmentation[b]
					ion type	theor m/z	exptl m/z	
1	tryptophan	$C_{11}H_{12}N_2O_2$	$[M + H]^+$ 205.09	4.03	$[M + H]^+$	205.0977	205.0997	**205**, 188, 146, 118, 91
					$[M - H]^-$	203.08205	203.0774	**203**, 142, 116, 74
2	4-methylthiobutyl glucosinolate	$C_{12}H_{23}NO_9S_3$	$[M - H]^-$ 420.05	3.89	$[M + K]^+$	460.0172	460.0112	**460**, 342, 238, 192
					$[M - H]^-$	420.0456	420.0459	**420**, 259, 178, 96
3	7-methylsulfinylheptyl glucosinolate	$C_{15}H_{29}NO_{10}S_3$	$[M - H]^-$ 478.09	3.87	$[M + K]^+$	518.05906	518.0777	**518**, 298
					$[M - H]^-$	478.08753	478.0866	**478**, 259, 96
4	indol-3-ylmethyl glucosinolate	$C_{16}H_{18}N_2O_9S_2$	$[M - H]^-$ 447.06	4.41	–	–	–	–
					$[M - H]^-$	447.05319	447.0525	**447**, 96
5	8-methylsulfinyloctyl glucosinolate	$C_{16}H_{31}NO_{10}S_3$	$[M - H]^-$ 492.10	4.74	$[M + Na]^+$	516.10077	516.094	**516**, 414, 252, 96
					$[M - H]^-$	492.10318	492.1010	**492**, 428, 96
6	5-methylthiopentyl glucosinolate	$C_{13}H_{25}NO_9S_3$	$[M - H]^-$ 434.06	5.01	–	–	–	–
					$[M - H]^-$	434.06132	434.0618	**434**, 96
7	4-methoxyindol-3-ylmethyl glucosinolate	$C_{17}H_{22}N_2O_{10}S_2$	$[M - H]^-$ 477.06	5.21	$[M + K]^+$	517.03529	517.0386	**517**, 479, 437, 399, 237, 160
					$[M - H]^-$	477.06376	477.0606	**477**, 96
8	dihydrocamalexic acid	$C_{12}H_{10}N_2O_2S$	$[M + H]^+$ 247.05	5.59	$[M + H]^+$	247.05412	247.0605	**247**, 201, 143, 118
					–	–	–	–
9	1-methoxyindol-3-ylmethyl glucosinolate	$C_{17}H_{22}N_2O_{10}S_2$	$[M - H]^-$ 477.06	5.91	$[M + H]^+$	479.07941	479.0876	**479**, 437, 399, 237, 160
					$[M - H]^-$	477.06376	477.0629	**477**, 44, 96
10	unknown aliphatic glucosinolate*	$C_{14}H_{26}NO_9S_2$	$[M - H]^-$ 416.10	6.60	–	–	–	–
					$[M - H]^-$	416.10489	416.1093	**416**, 389, 357, 323, 119, 96
11	7-methylthioheptyl glucosinolate	$C_{15}H_{29}NO_9S_3$	$[M - H]^-$ 462.09	6.76	–	–	–	–
					$[M - H]^-$	462.09261	462.0941	**462**, 96
12	8-methylthiooctyl glucosinolate	$C_{16}H_{31}NO_9S_3$	$[M - H]^-$ 476.11	7.55	$[M + H]^+$	478.12391	478.1278	**478**, 398, 236
					$[M - H]^-$	476.10826	476.1089	**476**, 96
13	3-(1,3-thiazol-2-yl)-1*H*-indole (camalexin)	$C_{11}H_8N_2S$	$[M + H]^+$ 201.05	8.91	$[M + H]^+$	201.04864	201.0517	**201**, 174, 160, 142, 59
					$[M - H]^-$	199.03299	199.0321	**199**, 158, 141, 130
14	5,7-dihydroxy-4′-methoxy-isoflavone (biochanin A)**	$C_{16}H_{12}O_5$	$[M + H]^+$ 285.07	10.93	$[M + H]^+$	285.07629	285.0773	**285**, 270, 242, 213, 152, 124
					$[M - H]^-$	283.06065	283.0594	**283**, 268, 239, 211, 132

[a]Note: dashes (−) denote missing ions, or when expected, pseudomolecular ions or adducts could not be identified. Rt, retention time; *, tentative annotation; and **, internal standard. [b]Numbers in bold represent precursor ions.

thaliana. Therefore, it had to be possible to unequivocally identify these metabolites in plant extracts. As reported previously,[18] reversed phase liquid chromatography is the best suited technique for the analysis of secondary metabolites in *Arabidopsis*. In this project, two C18 columns were assayed, a XTerra C18 and a ProntoSIL C18SH. Samples of stressed *A. thaliana* plants were analyzed in triplicate by using also two gradients. As expected, the XTerra column tested with either gradient 1 or gradient 2 gave worse resolution than ProntoSIL C18SH due to the higher particle size, although all considered metabolites could be detected. Using gradient 2, the total chromatographic run took about 20 min, and all glucosinolates and camalexin eluted in less than 11 min, and using gradient 1, the total chromatographic run took 30 min, and the metabolites of interest eluted within 15 min.

After the chromatographic analysis, peaks were extracted and aligned using xcms software. The number of peaks obtained was taken as an estimate of the performance of the column. In both cases, samples analyzed with the ProntoSIL C18SH column rendered a higher number of aligned mass features than the same samples analyzed with XTerra C18 LC-MS. The use of the short gradient implied losing 12.2 and 5.3% of the total peaks in positive and negative mode, respectively. This was considered acceptable taking into consideration the time (10

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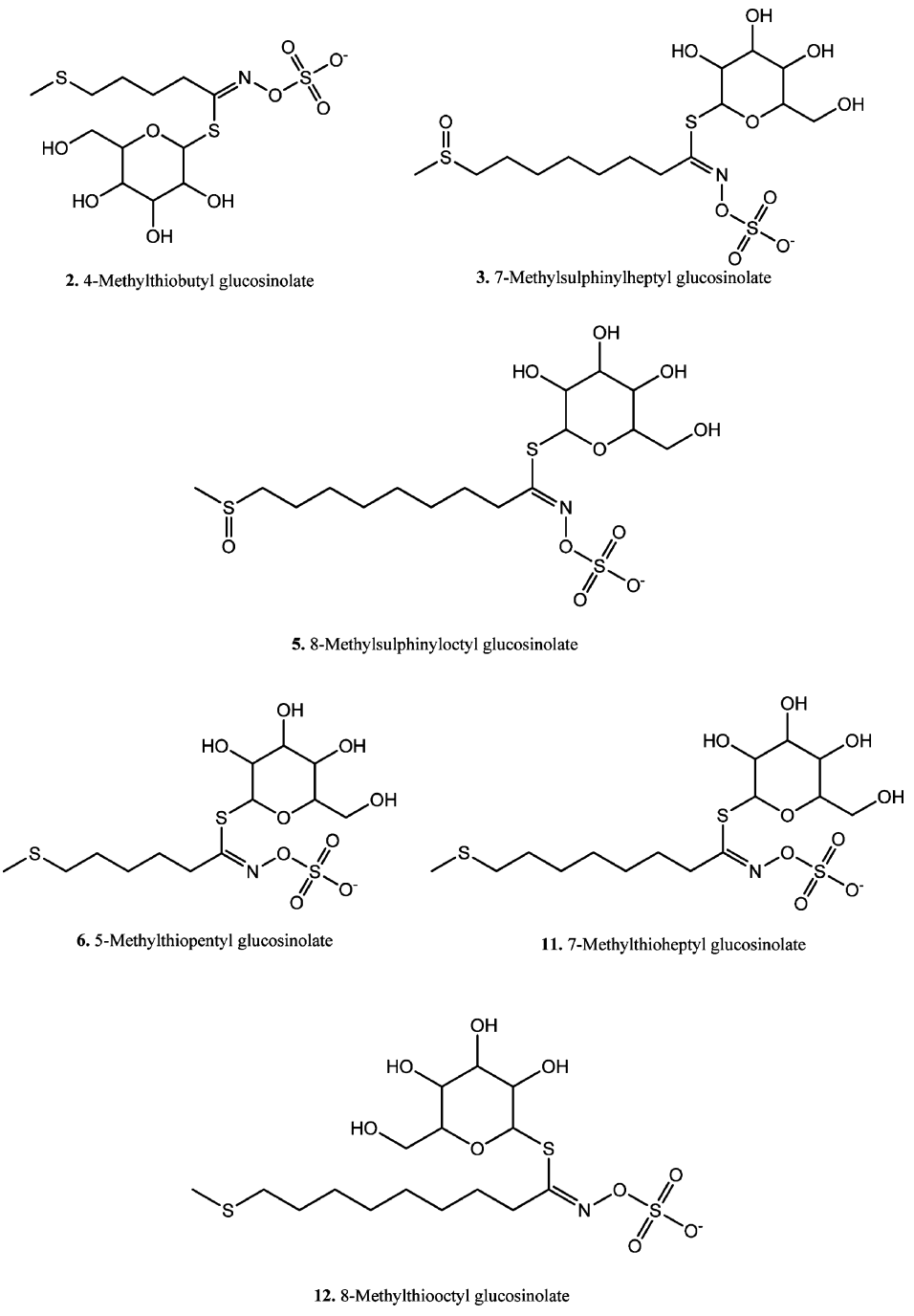

Figure 2. Structures of detected aliphatic glucosinolates.

min) saved in each analysis. Therefore, in the following analyses, the short gradient was chosen along with the ProntoSIL C18SH column.

Identification of Glucosinolates and Camalexin in Stressed *Arabidopsis* Mutants. The capability of QTOF-MS to measure masses with high accuracy makes this platform a suitable tool to perform nontargeted analysis. Therefore, characteristic fragmentation patterns allow the identification of glucosinolates and camalexin with a high degree of confidence without having to use pure standards. In the present study, typical fragmentation patterns were used to identify glucosinolates and camalexin. Moreover, the biological information of *Arabidopsis* mutants (*pad3*, *pen3.1*, and *cyp79B2/B3*) as well as Col-0 accessions contributed to the unequivocal identification of the analytes considered in this study. Ten glucosinolates, camalexin, and other related compounds were identified (Table 1).

Compounds **4**, **7**, and **9** were annotated as indolic glucosinolates, whereas compounds **2**, **3**, **5**,**6**, **11**, and **12** (Table 1) were tentatively annotated as aliphatic glucosinolates. On the basis of recent literature,[19] compound **10** was also tentatively annotated as an aliphatic glucosinolate with seven carbons in a linear or branched chain, the extent of which could not be properly determined with mass spectrometry data. With the chromatographic conditions used in this work, it was not possible to properly retain other previously reported aliphatic glucosinolates such as 3-methylsulfinylpropyl, 5-methylsulfinylpentyl, and 6-methylsulfinylhexyl. However, the methodology

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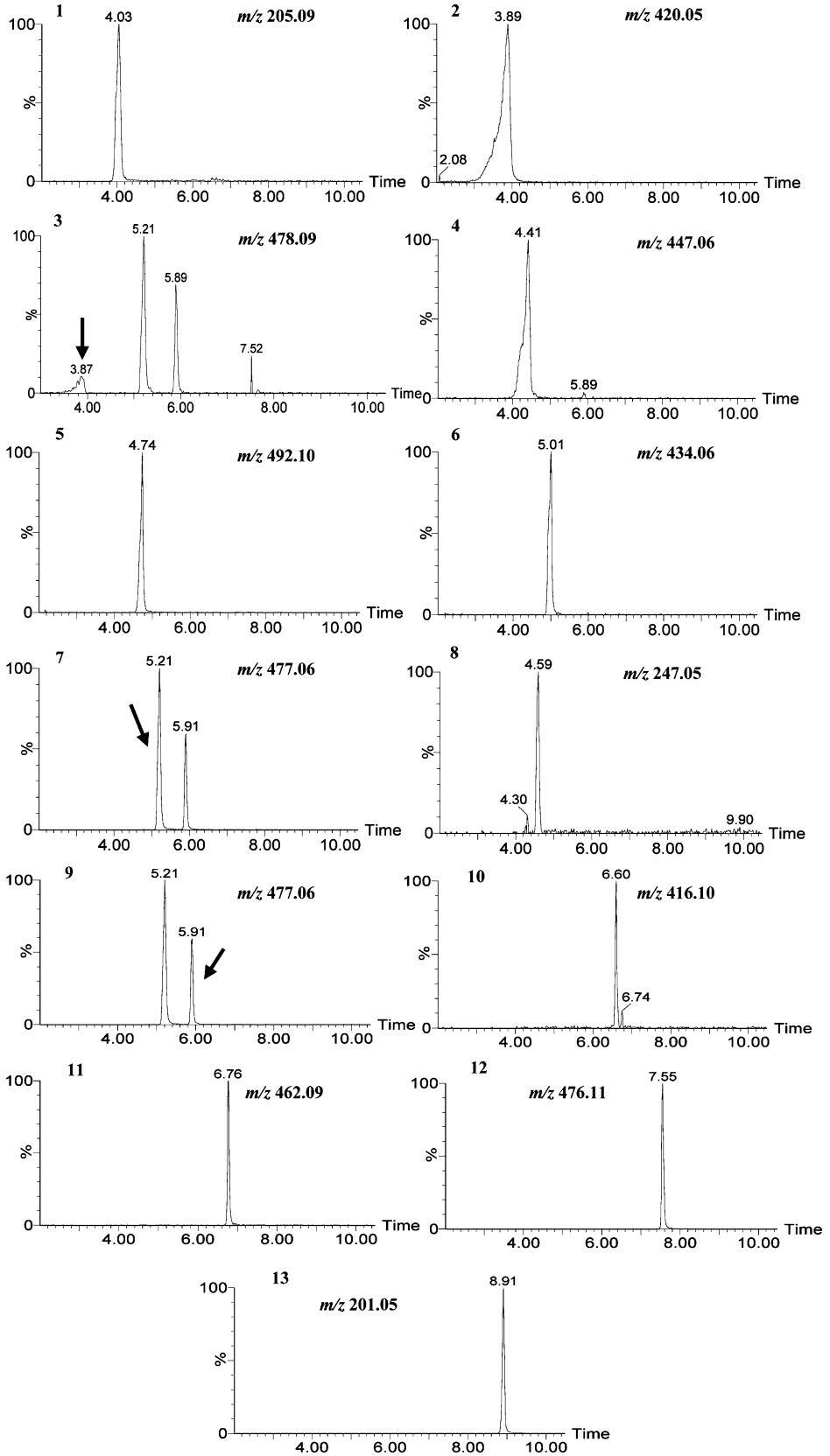

Figure 3. Extracted ion chromatograms for the 10 identified glucosinolates and camalexin. Values within each chromatogram represent m/z used for the extracted ion chromatogram.

proved to be sufficient to profile indolic glucosinolates. Related metabolites such as tryptophan (**1**) and dihydrocamalexic acid (**8**) were identified and annotated based on biological as well as mass spectrometric data. Extracted ion chromatograms for each compound are shown in Figure 3. As observed in chromatograms, both compounds **7** and **9** showed a maximum at 477.06

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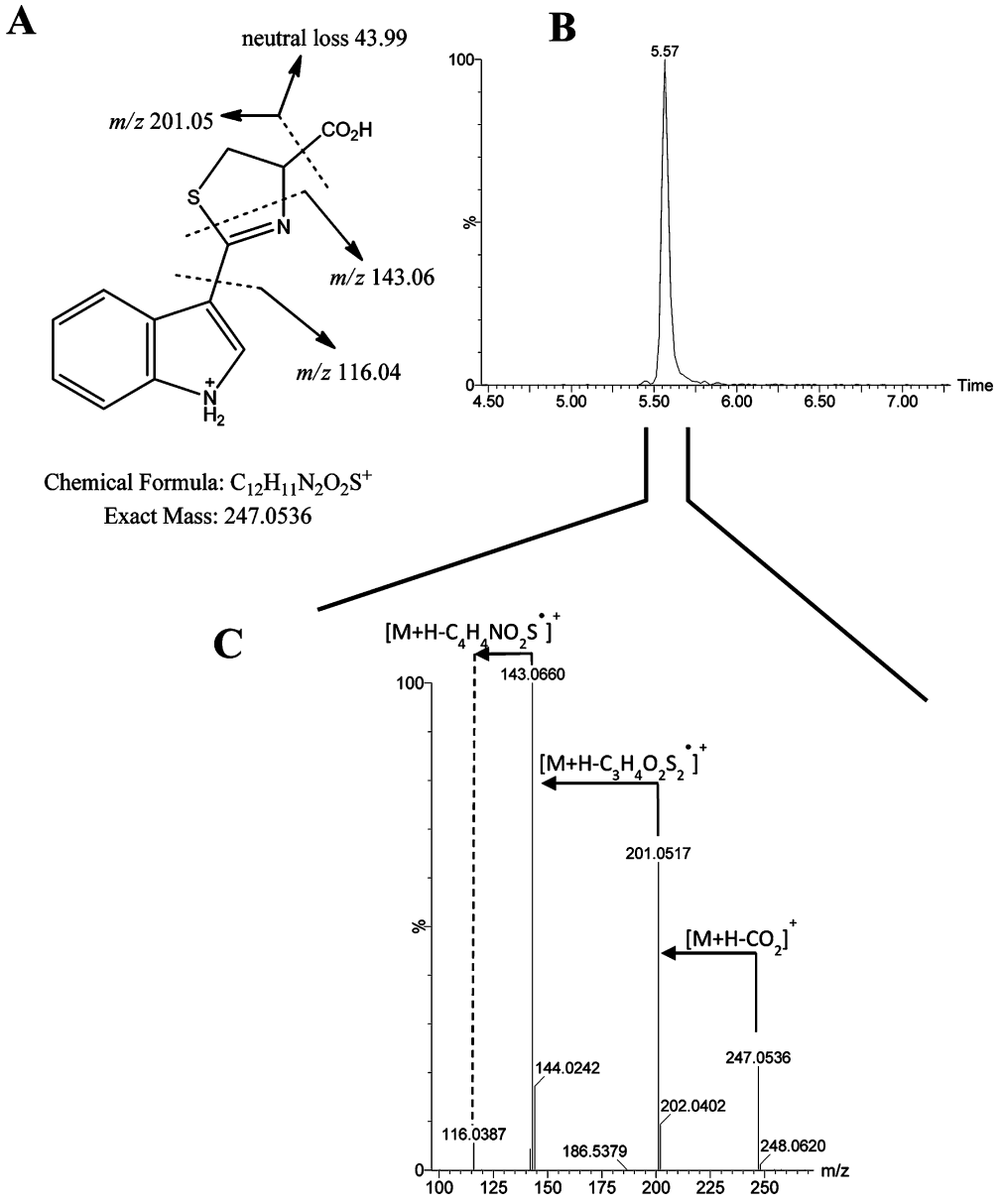

Figure 4. Identification of dihydrocamalexic acid by mass spectrometry: fragmentation pattern of dihydrocamalexic acid (A), chromatographic peak of ddihydrocamalexic acid (B), and fragment ions observed upon collision-induced dissociation (C).

corresponding to the $[M - H]^-$ of 4-methoxyindol-3-ylmethyl glucosinolate and the 1-methoxy isomer, respectively. Identification of each of the isomers was accomplished by their elution order.[19]

Characteristic fragmentation patterns were analyzed to confirm the identity of each compound. Table 1 shows the main fragments obtained from each extracted ion chromatogram from function 2 acquisition. The characteristic fragment peak at *m/z* 96.96 was observed in all recorded glucosinolate spectra representing a bisulphate anion (HSO_4^-) released after cleavage of intact glucosinolates. A chromatographic peak eluting at 2.0 min with a *m/z* of 463.05 compatible with the pseudomolecular ion ($[M - H]^-$) from 4-hydroxyindol-3-ylmethyl glucosinolate was observed, although its identity could not be properly confirmed due to the low signal intensity shown that rendered a poor fragmentation in function 2.

To verify these results, leaf extracts of stressed *Arabidopsis cyp79B2/B3* plants were analyzed by mass spectrometry. The *cyp79B2/B3* double mutant is a hybrid between two lines identified in a loss-of-function screening on a T-DNA insertion collection of the Col-0 ecotype. The T-DNA insertions disrupt their respective genes, rendering null alleles[24] and plants devoid in any indolic glucosinolate or camalexin.[22] In the original article,[24] the authors reported subtle phenotype differences between *cyp79B2/B3* double mutant and its respective wild-type Col-0; however, under the growth conditions used in this study, both sets of plants were completely indistinguishable (data not shown). As expected, indolic glucosinolates (**4**, **7**, and **9**) were not detected in any of these samples, whereas aliphatic glucosinolates (**2**, **3**, **5**, **6**, and **10**−**12**) were detected in *cyp79B2/B3* extracts. *Arabidopsis pad3*, isolated from an ethylmethanesulfonate mutant population,[23] carries a single nucleotide deletion, leading to an early stop codon in the predicted open reading frame that originates truncated mRNA, which is not translated into a functional CYP71B15 enzyme (that converts dihydrocamalexic acid into camalexin). Corre-

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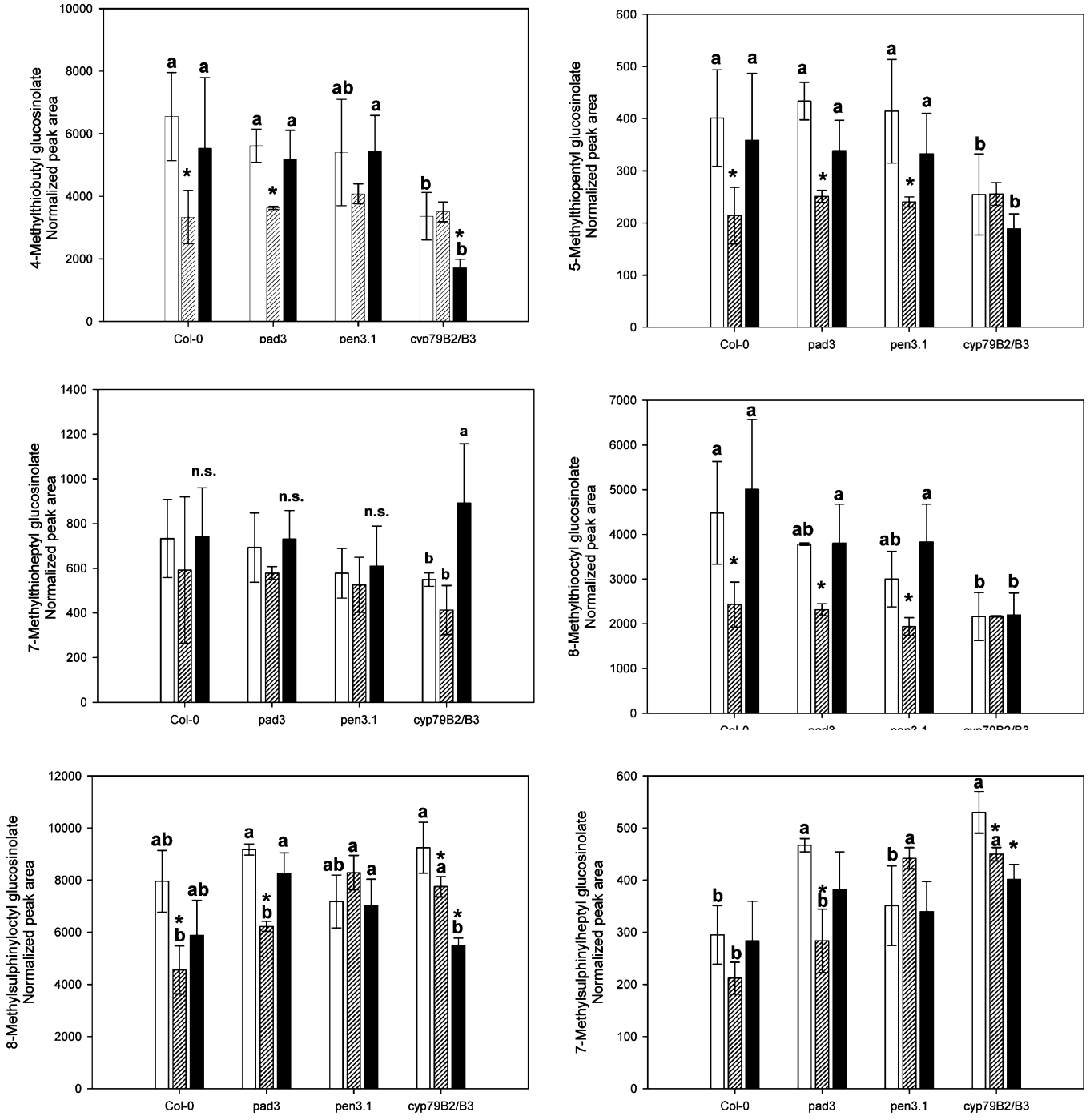

Figure 5. Relative quantitation of aliphatic glucosinolate levels in *Arabidopsis* rosette leaves in response to abiotic and biotic stresses. White bars represent control plants sprayed with tap water, striped bars represent plants sprayed with a *B. cinerea* conidia suspension, and black bars represent plants sprayed with a $AgNO_3$ solution. Asterisks (*) denote statistical significance at $P \leq 0.05$ with controls. Different letters denote statistical differences among genotypes subjected to the same treatment at $P \leq 0.05$; n.s., not significant.

spondingly, camalexin could not be detected either in *pad3* or in *cyp79B2/B3* leaf extracts. Nevertheless, a mass chromatographic feature showing a fragmentation pattern compatible with the presence of camalexin, an indolic ring, and neutral losses of H_2CO_2 and $C_3H_4O_2S$ was observed (Figure 4). This mass chromatographic feature was annotated as dihydrocamalexic acid, the immediate precursor of camalexin and substrate of phytoalexin-deficient-3 (PAD3) protein, by comparison with the reported data in the literature.[22] This metabolite was strongly accumulated in *pad3* plants after stress imposition (Figure 6).

Evaluation of the Stress Treatments. The impact of abiotic and biotic stress treatments on the analytes described above was evaluated after LC/ESI-QTOF-MS analysis. The relative quantitation was carried out first by determining recovery of the internal standard biochanin A (Table 1) to correct areas of target analytes. Finally, corrected peak areas were normalized to the amount of tissue used. Figure 5 shows

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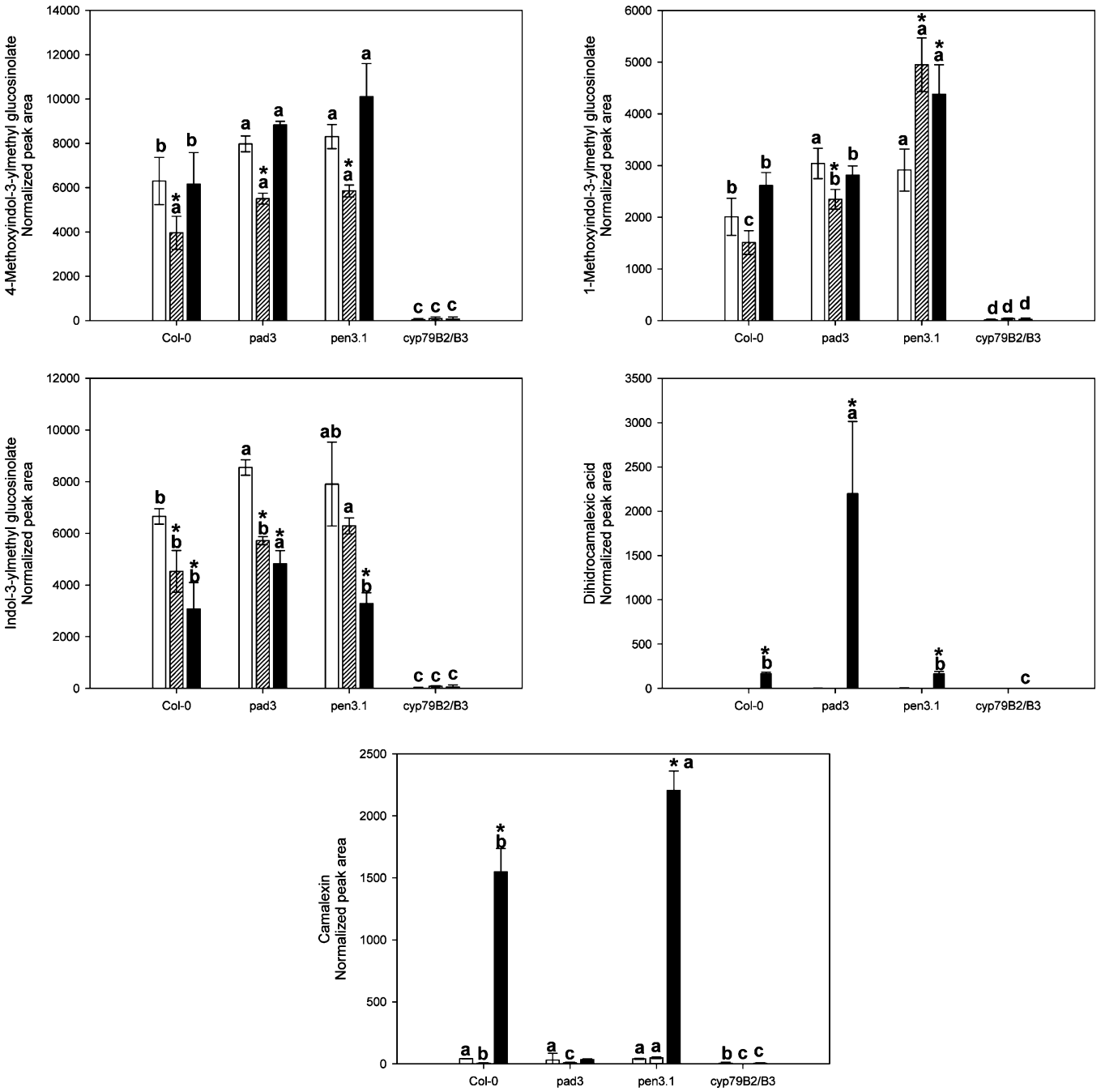

Figure 6. Relative quantitation of indolic glucosinolates, dihydrocamalexic acid and camalexin levels in *Arabidopsis* rosette leaves in response to abiotic and biotic stresses. White bars represent control plants sprayed with tap water, striped bars represent plants sprayed with a *B. cinerea* conidia suspension, and black bars represent plants sprayed with a $AgNO_3$ solution. Asterisks (*) denote statistical significance at $P \leq 0.05$ with controls. Different letters denote statistical differences among genotypes subjected to the same treatment at $P \leq 0.05$.

aliphatic glucosinolate concentrations in the different genotypes under control and stress conditions.

First, it should be noted that the different mutations had an effect on basal aliphatic glucosinolate levels. In general, *cyp79B2/B3* showed much reduced levels of aliphatics (over 50% for **2** and **12** and 40% for **6**). Nevertheless, both *pad3* and *cyp79B2/B3* showed higher basal levels of **3**, with respect to Col-0. Spraying *Arabidopsis* plants with a *Botrytis* conidia suspension reduced below control levels most aliphatic glucosinolates such as **2**, **6**, **5**, **12**, and **3** in Col-0 and *pad3*. The mutant *pen 3.1*, highly sensitive to biotic stress [*PEN3* encodes for an ATP binding cassette (ABC) transporter involved in the targeted export of toxins to penetration sites], exhibited a slightly different trend, since treatment with the fungus conidia increased **5** and **3** levels. In the double mutant, *cyp79B2/B3*, a different trend for **2**, **6**, and **12** was also observed with no significant differences with controls in response to the biotic elicitor. Levels of aliphatic glucosinolates did not significantly vary in *Arabidopsis* plants treated with $AgNO_3$ solution except in the case of *cyp79B2/B3* mutants. In this genotype, abiotic stress reduced levels of **2**, **6**, **5**, and **3** and increased the concentration of **11**.

Basal levels of indolic glucosinolates (Figure 6) were significantly higher in *pad3* and *pen 3.1*. This could be likely

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a feedback response to the constitutive absence of camalexin. In addition, an expected result was the complete absence of these metabolites in the *cyp79B2/B3* mutant as it is impaired in the activity that catalyzes the conversion of tryptophan to indolic-3-acetaldoxime (Figure 1). In addition, compounds **13** and **8** were absent in these plants after treatment with $AgNO_3$ or *Botrytis*. Moreover, also as predicted, *pad3* mutant failed to accumulate camalexin (**13**) upon stress treatment but over-accumulated dihydrocamalexic acid (**8**), the immediate metabolic precursor of camalexin.[28] In general, treatment with *Botrytis* conidia suspension reduced all indolic glucosinolates analyzed in Col-0 and *pad3*, whereas spraying with $AgNO_3$ did not change their levels with respect to controls. On the contrary, in *pen 3.1* plants, levels of **9** increased in response to biotic or abiotic elicitors with respect to controls, reflecting its higher sensitivity to both kinds of adverse conditions.[20] Levels of compound **13** significantly increased upon spraying with $AgNO_3$ solution in Col-0 and *pen 3.1* mutants, but they did not vary in response to *Botrytis* treatment. In addition, this metabolite could not be observed in *pad3* or *cyp79B2/B3* mutants. As expected, the dihydrocamalexic acid (**8**) concentration increased in $AgNO_3$-treated *pad3* plants instead (2000-fold with respect to control values) and to a much lower extent in Col-0 and *pen 3.1*, directly linking camalexin (**13**) production to the previous accumulation of dihydrocamalexic acid (**8**).

It has been shown that inoculation of *A. thaliana* with *B. cinerea* conidia reduces both aliphatic and indolic glucosinolate contents.[29] However, the apparent inconsistency of our data with previous reports showing that *B. cinerea*, a necrotrophic ascomycete, induces camalexin production and accumulation in *Arabidopsis* can be partially explained by the fact that some *B. cinerea* isolates are camalexin-tolerant and capable of detoxifying this phytoalexin.[29]

Differences among *Arabidopsis* Mutants under Control Conditions. The complete data set of each *Arabidopsis* genotype was analyzed by PCA (Figure 7). The first two principal components explained 43.6% of the total variance in negative mode and 53.0% in positive mode. As extracted from the PCA plots, principal component 1 (PC1) explained the experimental variation associated with this kind of experiment and clearly differentiated the two biological replicates, whereas PC2 was associated with genotype-specific variation. Hence, PC2 clearly discriminated Col-0, *pad3*, and *pen3.1* genotypes from the *cyp79B2/B3* genotype. The similarity among Col-0, *pad3*, and *pen3.1* genotypes allowed us to conclude that the metabolic differences found among these genotypes only appeared after stress imposition. On the contrary, clear basal differences were found between any genotype and the double mutant *cyp79B2/B3*. This genotype carries insertions in the genes coding for the enzymes that catalyze the conversion of tryptophan to indole-3-acetaldoxime, the first metabolite in the indolic glucosinolates biosynthetic pathway, which also acts as a precursor for camalexin biosynthesis.[30] To find out which variables (metabolites) were behind these basal differences, a PLS-DA analysis was performed. Among these variables, it was found that indolic glucosinolates were important because they were absolutely absent in *cyp79B2/B3* samples, as expected. Strikingly, tryptophan (**1**) was also found to be important in defining these differences. Relative quantitation of **1** in control *cyp79B2/B3* was conducted and expressed in Figure 8. The results obtained suggested that as a result of the metabolic deficiency in cytochromes 79B2 and 79B3, the precursor metabolite tryptophan (**1**) showed an accumulation of 4-fold with respect to the rest of genotypes included in this study. These results also suggested that no negative feedback mechanism prevented tryptophan from accumulating in leaf rosettes of *cyp79B2/B3* plants. In addition, no specific phenotype was observed (data not shown): leaf rosette and inflorescence phenotype as well as development were identical to that of Col-0 wild type, indicating that tryptophan overaccumulation had no negative effects on plant performance.[24]

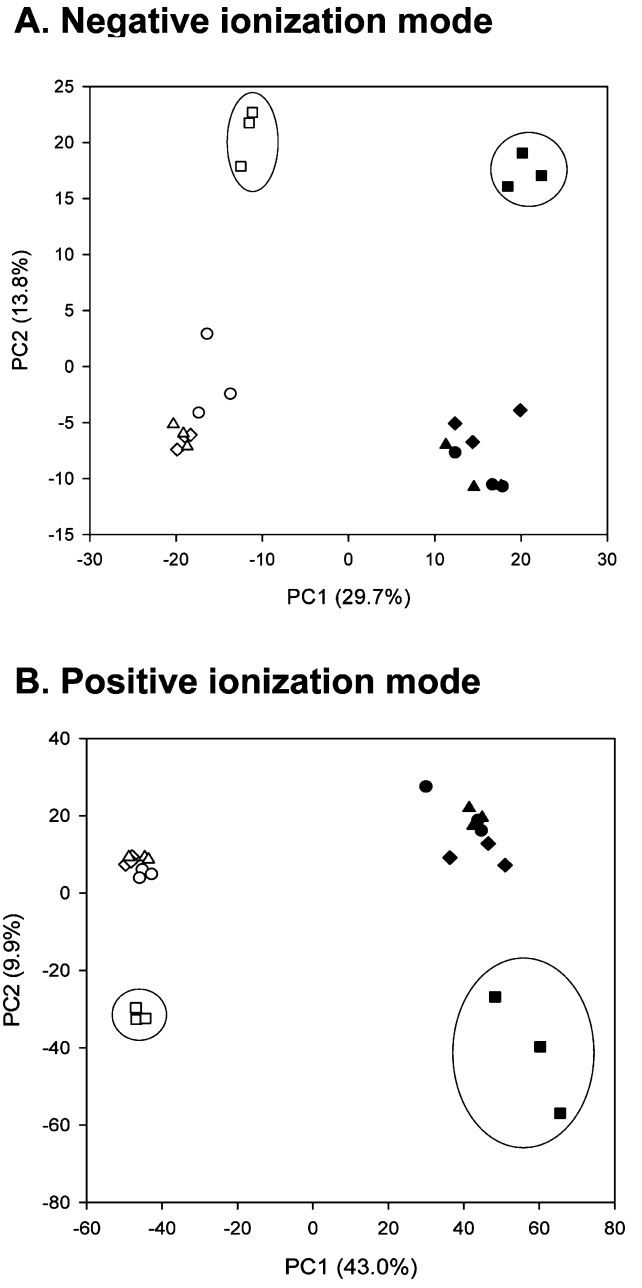

Figure 7. PCA of data sets belonging to *Arabidopsis* Col-0 (wild type) and *cyp79B2/B3*, *pad3*, and *pen3.1* mutant leaf extracts under control conditions. Plots represent the PCA analysis in negative (A) and positive (B) ionization modes. Symbols in black and white belong to *Arabidopsis* accessions in replicates 1 and 2, respectively. Refer to Col-0 (○), *cyp79B2/B3* (□), *pad3* (◇), and *pen3.1* (△). Circles indicate *cyp79B2/B3* sample groups in both negative and positive modes and in the two biological replicates.

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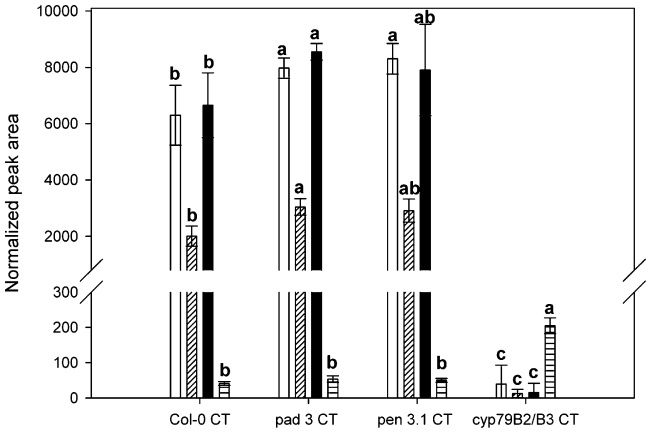

Figure 8. Relative quantitation of tryptophan and indolic glucosinolate levels in *Arabidopsis* rosette leaves under control conditions. White bars represent compound **7** levels, bars with diagonal lines represent compound **9** levels, black bars represent compound **4** levels, and bars with horizontal lines represent compound **1** levels. Different letters denote statistical differences among genotypes subjected to the same treatment at $P \leq 0.05$.

Overall, data presented in this work confirm the use of reversed phase liquid chromatography coupled to QTOF-MS as a useful methodology to profile semipolar compounds in plant extracts, especially glucosinolates and other defense-related compounds. In addition, the use of mutants carrying alterations in certain biosynthetic pathways in combination with mass spectrometry could be useful in the unequivocal identification of compounds for which commercial standards are unavailable. Both the inoculation with *B. cinerea* conidia and the treatment with a $AgNO_3$ solution have an effect on secondary metabolism but not in the same direction, whereas the biotic elicitor depresses glucosinolate content without any direct effect on camalexin production, the abiotic stress treatment does not alter aliphatic or indolic glucosinolate contents but induces the accumulation of the phytoalexin camalexin. In addition, the impairment in PEN3 (PDR8) ATP-binding cassette transporter in the mutant *pen 3.1* has a positive effect on indolic glucosinolate and camalexin contents in response to biotic or abiotic elicitation and even in nonstressed plants, pointing out to the higher sensitivity of this genotype to stress. Finally, the impairment in cytochrome P79 enzyme activity seems to induce specific alterations in secondary metabolite composition even under nonstressful condition (such as the accumulation of tryptophan). Therefore, because the basal metabolic configuration is different to Col-0, their comparison in genetic and physiological studies should be taken with caution.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of chromatographic columns tested and characteristics and number of peaks collected in the two gradients used and figure of total ion current chromatograms of plant extracts for the different columns and gradients assayed in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

PCA, principal component analysis; PLS-DA, partial least-squares discriminant analysis

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