A rapid and sensitive method for the simultaneous analysis of aliphatic and polar molecules containing free carboxyl groups in plant extracts by LC-MS/MS

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Abstract

Background: Aliphatic molecules containing free carboxyl groups are important intermediates in many metabolic and signalling reactions, however, they accumulate to low levels in tissues and are not efficiently ionized by electrospray ionization (ESI) compared to more polar substances. Quantification of aliphatic molecules becomes therefore difficult when small amounts of tissue are available for analysis. Traditional methods for analysis of these molecules require purification or enrichment steps, which are onerous when multiple samples need to be analyzed. In contrast to aliphatic molecules, more polar substances containing free carboxyl groups such as some phytohormones are efficiently ionized by ESI and suitable for analysis by LC-MS/MS. Thus, the development of a method with which aliphatic and polar molecules -which their unmodified forms differ dramatically in their efficiencies of ionization by ESI- can be simultaneously detected with similar sensitivities would substantially simplify the analysis of complex biological matrices.

Results: A simple, rapid, specific and sensitive method for the simultaneous detection and quantification of free aliphatic molecules (e.g., free fatty acids (FFA)) and small polar molecules (e.g., jasmonic acid (JA), salicylic acid (SA)) containing free carboxyl groups by direct derivatization of leaf extracts with Picolinyl reagent followed by LC-MS/MS analysis is presented. The presence of the N atom in the esterified pyridine moiety allowed the efficient ionization of 25 compounds tested irrespective of their chemical structure. The method was validated by comparing the results obtained after analysis of Nicotiana attenuata leaf material with previously described analytical methods.

Conclusion: The method presented was used to detect 16 compounds in leaf extracts of N. attenuata plants. Importantly, the method can be adapted based on the specific analytes of interest with the only consideration that the molecules must contain at least one free carboxyl group.

Background

The analysis of low abundant signalling molecules such as phytohormones (e.g., jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA)) and intermediates of metabolic pathways (e.g., free fatty acids (FFA), oxygenated forms of fatty acids) in plants is an important tool to understand how plants grow, develop and respond to stress conditions. For high-throughput biochemical phe-
Ionization by electrospray (ESI) is one of the most widely used tools for LC-MS/MS analysis, and the ions can be selectively monitored in negative and positive mode [2]. In the ESI negative mode, analysis of small molecules containing free carboxyl groups, yields mainly the ion [M-H], corresponding to their carboxylate anion. However, the efficiency of formation of carboxylate anions differs widely among compounds depending on their chemical structure [3]. For example, while small molecules containing free carboxyl groups and high numbers of heteroatoms such as the phytohormones JA, SA and ABA or aromatic substances are ionized efficiently by ESI [4-6], aliphatic molecules such as FFAs are relatively poorly ionized by this technique, in particular, as the aliphatic chain becomes longer and the degree of saturation higher [7]. This lower efficiency of carboxylate anion formation restricts the analysis of aliphatic molecules, specially, when small amounts of tissue are available for analysis (e.g., embryos, pollen, tissue sections obtained by laser micro-dissection).

Analytical methods to quantify aliphatic molecules are laborious, involving enrichment steps using chromatographic techniques such as thin layer chromatography (TLC), solid phase extraction (SPE) or LC previous to derivatization and gas chromatography (GC) for separation and analysis [8]. One strategy to improve the sensitivity of analysis of aliphatic molecules containing carboxyl groups by LC-MS/MS is the use of chemical derivatization that generates a strong ion in the ESI source [8]. Among these, the generation of pyridinium compounds has many advantages [9]. The presence of the N atom in the pyridine moiety allows for the efficient ionization of the compounds [9] and due to the mild conditions used to generate the Picolinyl ester intermediates, analysis of sensitive molecules containing conjugated double bonds is possible [10]. Moreover, since ester bonds are resistant to the conditions used to generate Picolinyl ester derivatives, esterified fatty acids do not interfere with the analysis [10].

In this study, a method for the simultaneous determination of aliphatic molecules (e.g., FFAs and their oxygenated derivatives) and more polar (phytohormones, phenolics) compounds by using Picolinyl ester derivatives of leaf extracts coupled with LC-MS/MS is presented. The method was applied for the analysis of leaves of *N. attenuata* plants before and after wounding and elicitation by the insect elicitors fatty acid-amino acid conjugates (FACs) [11]; which are treatments known to stimulate the production of a large number of phytohormones and secondary metabolites [12].

Results and Discussion

Method development

Commercial chemical standards (Table 1) were first derivatized to their respective Picolinyl esters using the mild method proposed in [10]. Some examples of the molecules used are shown in Figure 1. This method allows the quantitative derivatization of free carboxyl group-containing molecules within 10 min using conditions that preserve sensitive molecules containing conjugated double bonds. The first step in the reaction involves the activation of the carboxyl group with 1,1′-carbonyldiimidazole to form an active carbimidazol amid (1, Figure 2). The second step involves the reaction of 1 with 3-(hydroxymethyl)-pyridine to form the corresponding β-Picolinyl ester (2, Figure 2).

As mentioned in the background section, the formation of Picolinyl esters by this method is restricted to free carboxyl groups [10]. To confirm the absence of hydrolysis of ester bonds from esterified fatty acids, 10 µg of commercial glycerolipids (monogalactosyldiglycerol: MGDG, phosphatidylcholine: PC and phosphatidylglycerol: PG) were first purified by TLC and then subjected to the reaction. No free fatty acids could be detected (data not shown and see below for the methodology used for detection), confirming that the reaction does not induce the hydrolysis of esterified fatty acids.

Analysis of the Picolinyl ester derivatives of the commercial standards was first accomplished by their direct injection into the MS-interface to determine their [M+H]+ parent ion and their MS/MS fragmentation pattern by collision induced dissociation (CID) using increasing voltage energies. The third mass analyzer was set in the scan mode for ions with *m/z* between 50 and 500. Two major frag-
ments were generated, \( m/z = 92 \) and \( m/z = 108 \), resulting from the loss of the methyl-pyridine fragment and the hydroxymethyl-pyridine fragment, respectively (Figure 2). The ion \( m/z = 92 \) gave the strongest intensity at a collision energy of -25.5 V and therefore the \([M+H]^+ > 92\) transition was used for specific detection of Picolinyl ester derivatives.

The chromatographic separation of Picolinyl ester derivatives was performed on a reverse-phase column using acidic water and methanol as solvents in a gradient mode. In this case, the LC method was optimized for the analysis in plant extracts of small polar molecules and aliphatic molecules containing no more than 18 carbons. After a pre-run of 1.5 min, all substances of interest eluted from the column in 18.5 min. An additional post run of 6.5 min was added for column conditioning for a final run time of 25 min. An example of chromatograms (total ion current, TIC) for derivatized commercial standards and a derivatized leaf extract from \( N. \) attenuata is shown in Figures 3A and 3B, respectively.

**Figure 1**
Examples of compounds analyzed as their Picolinyl ester derivatives by LC-MS/MS.
Mixtures of derivatized commercial standards ranging from 100 to 1000 pg/μL were first used to determine their linear range of detection and their limit of detection (LOD). These concentrations were in the same range as the endogenous compounds quantified in derivatized leaf extracts (see below). Within this range, most analytes presented a linear response (concentration vs. area) with r values higher than 0.97 with the exception of ICA, GA3 and 2H2-OPC 8:0 which presented r values between 0.93 and 0.94 (Table 1). The LODs, calculated based on the calibration plot method, were between 5 and 42 pg/μL (Table 1). To determine the injection precision, each derivatized commercial standard was injected 10 times at different concentrations and the coefficient of variation (CV) was calculated. For all compounds, the CV values were less than 0.1 for all concentrations tested (Additional file 1, Table S1).

Table 1: List of standards used for matrix free and matrix adapted calibration.

| Number | Substance | [M+H]⁺ Retention time [min] | Linearity (r) $^\S$ | LOD [pg/μL] | Linearity (r) $^\S$ | LOD [pg/μL] |
|--------|-----------|-----------------------------|---------------------|--------------|---------------------|--------------|
| 1      | $^{13}$C₆-jasmonic acid-isoleucine ($^{13}$C₆-JA-Ile) | 421 6.86 | 1.00 | 20.60 | 0.99 | 116 |
| 2      | Jasmonic acid-isoleucine (JA-Ile) | 415 6.86 | 1.00 | 20.80 | 0.99 | 117 |
| 3      | $^{2}$H₄-salicylic acid ($^{2}$H₄-SA) | 234 8.77 | 0.98 | 24.40 | 0.98 | 167 |
| 4      | Salicylic acid (SA) | 230 8.77 | 0.97 | 24.80 | 0.98 | 165 |
| 5      | $^{2}$H₄-abscisic acid ($^{2}$H₄-ABA) | 362 9.01 | 0.98 | 19.40 | 0.98 | 163 |
| 6      | Abscisic acid (ABA) | 356 9.02 | 0.99 | 18.70 | 0.98 | 165 |
| 7      | Indole-3-carboxylic acid (ICA) | 253 9.08 | 0.93 | 41.30 | 0.91 | 355 |
| 8      | Royal jelly acid (Tr IS) | 278 9.26 | 0.99 | 12.00 | 0.98 | 181 |
| 9      | Cinnamic acid (CA) | 240 9.34 | 0.99 | 23.90 | 0.97 | 203 |
| 10     | Jasmonic acid (JA) | 302 9.43 | 0.98 | 19.60 | 0.98 | 155 |
| 11     | Traumatol | 306 9.85 | 0.99 | 12.90 | 0.98 | 174 |
| 12     | Indole-3-acetic acid (IAA) | 267 9.94 | 0.98 | 19.30 | 0.98 | 297 |
| 13     | Traumatin | 304 9.96 | 1.00 | 5.30 | 0.98 | 168 |
| 14     | 9,10-$^{2}$H₂-dihydro-jasmonic acid (D₂-JA) | 306 10.13 | 1.00 | 11.30 | 0.98 | 180 |
| 15     | Traumatic acid | 320 10.15 | 0.99 | 11.20 | 0.96 | 250 |
| 16     | Hexadecatrienoic acid (16:3) | 342 10.29 | 0.99 | 17.00 | 0.99 | 132 |
| 17     | (9 S, 13 S)-12-oxo-phytodienoic acid (OPDA) | 384 10.89 | 1.00 | 10.30 | 0.98 | 147 |
| 18     | Hexadecadienoic acid (16:2) | 344 10.93 | 0.99 | 14.90 | 0.98 | 147 |
| 19     | Gibberellin A₃ (GA₃) | 438 11.27 | 0.93 | 42.30 | 0.96 | 246 |
| 20     | $^{2}$H₂-OPC 8:0 | 388 11.59 | 0.94 | 0.97 | 0.96 | 0.97 |
| 21     | Linolenic acid (18:3) | 370 12.83 | 1.00 | 7.80 | 1.00 | 70 |
| 22     | Hexadecenoic acid (16:1) | 346 13.20 | 0.99 | 19.00 | 0.99 | 95 |
| 23     | Heptadecenoic acid (17:1) | 360 13.78 | 1.00 | 4.10 | 0.97 | 195 |
| 24     | Linoleic acid (18:2) | 372 14.22 | 0.97 | 8.60 | 0.96 | 123 |
| 25     | Hexadecanoic acid (16:0) | 348 14.53 | 1.00 | 5.20 | 0.99 | 114 |
| 26     | Octadecenoic acid (18:1) | 374 14.86 | 0.99 | 14.70 | 0.99 | 94 |
| 27     | Heptadecanoic acid (17:0) | 362 16.09 | 1.00 | 7.50 | 0.99 | 119 |
| 28     | Stearic acid (18:0) | 376 17.59 | 1.00 | 7.90 | 0.99 | 117 |

*¹ Amount of standard unknown.
² Matrix free calibration
³ Matrix adapted calibration
$^\S$Standard calibration curves were generated by injecting 10 μL of 100, 250, 375, 500, 750, 1000 pg/μL of the different standards (n = 3).

To determine matrix suppression effects in a leaf extract, mixtures of derivatized standards were spiked at different concentrations in underivatized leaf extracts of N. attenuata plants and the linear range of detection and LOD were calculated (Table 1). In this case, the linearity of the response (r value, concentration vs. area) was similar to that presented by the derivatized standards in pure solvent, however, the LOD values were increased for all standards to amounts between 70 and 350 pg/μL.

**Extraction and analysis of N. attenuata leaves**

The extraction of polar and lipophilic compounds from N. attenuata leaves was performed as indicated by [13] with the modifications adopted by [14]. Additional modifications were included to increase the number of samples that can be processed simultaneously and to reduce...
the amount of sample material necessary for analysis (see Methods section).

The extraction method was first validated by performing 10 biological replicates of *N. attenuata* non-elicited and FAC-elicited leaves after 60 min of the treatment. For each replicate, 300 mg of leaf tissue were extracted, derivatized and analyzed by LC-MS/MS (Additional file 1, Table S2). The standard deviations were below 10% of the average values for all detectable compounds. To determine the extraction recovery rate, the residual leaf material obtained after the first extraction was re-extracted, derivatized and analyzed. The recovery rates were higher than 98% for all molecules tested (Additional file 1, Table S2). Sample stability was tested by re-analyzing the derivatized leaf extracts after two days of the first analysis (samples were kept at 10°C). The amounts of all compounds did not differ significantly between the first and second analyses (paired *t*-test, data not shown). The CV was analyzed for the detectable compounds in derivatized leaf extracts (analyzed the same day of extraction) from FAC-elicited leaves (60 min after the treatment). The CV values were higher than 98% for all molecules tested (Additional file 1, Table S2). Sample stability was tested by re-analyzing the derivatized leaf extracts after two days of the first analysis (samples were kept at 10°C). The amounts of all compounds did not differ significantly between the first and second analyses (paired *t*-test, data not shown). The CV was analyzed for the detectable compounds in derivatized leaf extracts (analyzed the same day of extraction) from FAC-elicited leaves (60 min after the treatment). The CV values of two-day old extracts (kept at 10°C) were also analyzed. The CV values were below 0.1 for all detectable compounds (Additional file 1, Table S1). Finally, the efficiency of derivatization was evaluated by the analysis of the respective free compounds in derivatized leaf extracts (60 min after FAC elicitation). No free molecules were detected, corroborating that the derivatization is quantitative [10].

To validate the results obtained with the method presented in this study, 300 mg of non-elicited leaves of *N. attenuata* plants and leaves wounded and elicited with FAC (30 and 60 min) were analyzed with the present method and additionally with well established analytical methods for aliphatic compounds and phytohormones. Aliphatic compounds (free fatty acids in this case) were analyzed by performing separation by TLC and GC-MS analysis of their methyl-ester derivatives (see Methods section) and phytohormones were analyzed by LC-MS/MS using underivatized leaf extracts [6].

A total of 25 compounds were analyzed, including FFAs, derivatives and intermediates of the lipoxygenase pathway (e.g., OPDA, dnOPDA, OPCs) and some phytohormones related to stress responses or growth (e.g., JA, SA, ABA, JA-Ile, GAs, IAA). The analysis showed that 16 compounds could be detected in derivatized *N. attenuata* leaf extracts after elicitation. ICA, CA, traumatol, dnOPDA, IAA, 16:2, GA$_4$, OPC-8:0 and GA$_4$ could not be detected by any of the methods used (Table 2). The phytohormones IAA, GA$_4$, and GA$_4$ accumulate to low levels in leaf tissue and their detection usually requires purification steps. Thus, these molecules were most likely below their LOD.
Example of chromatograms of Picolinyl ester derivatives from a standard mixture and a *N. attenuata* leaf extract. A, Chromatogram (TIC) of a mix of derivatized commercial standards. B, Chromatogram (TIC) of a derivatized *N. attenuata* leaf extract after 60 min of FAC elicitation. *Analytes 11 to 13 are overlaid by a peak corresponding to an unknown compound in the leaf extract. Peaks are numbered according to Table 1.
Table 2: Quantification of Picolinyl ester derivatives of *N. attenuata* leaf extracts and comparison with two additional analytical methods.

| Substance     | RT    | Picol. esters Method | Picol. esters Method | Picol. esters Method | Picol. esters Method | Picol. esters Method | Picol. esters Method | Picol. esters Method | Picol. esters Method | Picol. esters Method | Picol. esters Method |
|---------------|-------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
|               |       | 0 min (± SE)         | 30 min (± SE)        | 60 min (± SE)        | 0 min (± SE)         | 30 min (± SE)        | 60 min (± SE)        | 0 min (± SE)         | 30 min (± SE)        | 60 min (± SE)        | 0 min (± SE)         |
|               |       | ng gFW^{-1}          | ng gFW^{-1}          | ng gFW^{-1}          | ng gFW^{-1}          | ng gFW^{-1}          | ng gFW^{-1}          | ng gFW^{-1}          | ng gFW^{-1}          | ng gFW^{-1}          | ng gFW^{-1}          |
| JA-ile        | 6.86  | 415                  | -                    | 1.1 (± 0.14)         | 39 (± 4.0)           | 46 (± 4.8)           | 92 (± 6.0)           | 81 (± 2.0)           | 83 (± 9.0)           | 97 (± 4.0)           | 158 (± 16)          |
| SA            | 8.77  | 230                  | 193 (± 31)           | 173 (± 24)           | 203 (± 19)           | 212 (± 15)           | 182 (± 11)           | 196 (± 22)           | 216 (± 17)           | 189 (± 18)           | 129 (± 19)          |
| ABA           | 9.01  | 356                  | 257 (± 7)            | 225 (± 35)           | 192 (± 9.0)          | 246 (± 14)           | 274 (± 6.0)          | 232 (± 10)           | 264 (± 12)           | 242 (± 14)           | 277 (± 14)          |
| ICA           | 9.08  | 253                  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    |
| CA            | 9.34  | 240                  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    |
| JA            | 9.43  | 302                  | 137 (± 20)           | 152 (± 150)          | 937 (± 95)           | 845 (± 125)          | 1845 (± 110)         | 1586 (± 180)         | 1564 (± 42)          | 1326 (± 350)         | 3820 (± 323)         |
| Traumatol     | 9.85  | 306                  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    |
| dnOPDA        | 9.85  | 356                  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    |
| IAA           | 9.94  | 267                  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    |
| Traumatol     | 9.96  | 304                  | 11.3 (± 5.6)         | 14.4 (± 7.8)         | 17.1 (± 4.8)         | 15.2 (± 2.3)         | 14.4 (± 3.4)         | 12.4 (± 2.7)         | 13.8 (± 1.9)         | 15.0 (± 2.6)         | 10.7 (± 1.6)         |
| Tr. acid**    | 10.15 | 320                  | -                    | -                    | 19.6 (± 0.96)        | 24.3 (± 3.2)         | 32.8 (± 6.7)         | 20.4 (± 6.1)         | 17.1 (± 2.5)         | 19.6 (± 1.9)         | 20.9 (± 4.2)         |
| OPC4:0        | 10.46 | 330                  | -                    | -                    | -                    | -                    | -                    | 12.6 (± 8.1)         | -                    | 10.3 (± 4.9)         | -                    |
| OPC6:0        | 10.67 | 358                  | -                    | -                    | -                    | -                    | -                    | 5.3 (± 2.9)          | -                    | 8.6 (± 5.6)          | -                    |
| OPDA          | 10.89 | 384                  | 107 (± 60)           | 91 (± 53)            | 323 (± 45)           | 386 (± 15)           | 291 (± 11)           | 322 (± 15)           | 180 (± 15)           | 184 (± 31)           | 301 (± 13)          |
| GA_{1}        | 11.27 | 438                  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    |
| OPC8:0        | 11.41 | 386                  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    |
| GA_{2}        | 11.99 | 424                  | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    | -                    |

Quantification of compounds was performed in triplicate (n = 3)

*: below limit of detection under the conditions tested

*: Method 2: Analysis of underivatized molecules by LC-MS/MS, this method was used to quantify compounds in the upper list section

*: Method 3: Analysis of methyl esters of FFA (FAMES) by GC-MS

** Tr. acid: Traumatic acid
Seeds of the 30th generation of an inbred line of high-throughput biochemical screenings. multiple samples quickly, making it compatible with tionally, the rapid extraction procedure without addi-
and multiple analytical techniques cannot be used. Addi-
amounts are limiting (e.g., embryos, ovules, pollen or spe-
This method may prove useful in cases in which tissue
method can be adapted to the needs of the investigator.
only a selected number of molecules were tested, the
Correlation analysis of underivatized extracts. For the remaining com-
ounds, a good correlation was observed between the
methods used (Table 2).
Finally, increasing amounts of leaf material (5 to 200 mg;
weight) were also extracted from N. attenuata plants
after 60 min of FAC elicitation to determine the range of
tissue amounts in which a linear correlation with the
amounts of detectable compounds was observed. The
results showed a linear correlation between the amount of
11 compounds and the amount of leaf material extracted
(figure 4), indicating that as little as 5 mg of leaf tissue
was sufficient for the reliable quantification of these mol-
cules. In the case of JA-Ile, at least 50 mg of leaf tissue
were required for detection while for OPC-6:0, OPC-4:0,
traumatin and traumatic acid more than 100 mg of tissue
were required (Additional file 1, Table S3).

Conclusion
A method was developed that enables the rapid, specific
and simultaneous analysis of aliphatic compounds such
as free fatty acids and small polar compounds such as phy-
tohormones in N. attenuata leaves. Although in this study
only a selected number of molecules were tested, the
method can be adapted to the needs of the investigator.
This method may prove useful in cases in which tissue
amounts are limiting (e.g., embryos, ovules, pollen or spe-
cific tissue sections obtained by laser micro-dissection)
and multiple analytical techniques cannot be used. Addi-
tionally, the rapid extraction procedure without addi-
tional purification steps may also facilitate the analysis of
multiple samples quickly, making it compatible with
high-throughput biochemical screenings.

Methods
Plant material and treatments
Seeds of the 30th generation of an inbred line of Nicotiana attenuata plants were used as the wild-type genotype (WT)
in all experiments. Plants were grown as described in [15].
For all experiments, leaves at nodes +1 of rosette stage
(40-day old) plants were used. Wounding was performed
by rolling three times a fabric pattern wheel on each side
of the midvein. For FAC treatment, the wounds were
immediately supplied with 20 μL of synthetic N-lino-
lenoyl-glutamic acid (18:3-Glu; 0.03 nmol/μL in 0.02%
(v/v) Tween-20/water). Leaf tissue was collected at 30 and
60 min after the treatments and frozen immediately in liq-
uid nitrogen for subsequent analysis. Non-elicited leaf tis-
ue was collected without any previous treatment.

Chemicals
Chloroform, dichloromethane, methanol and hexane
were from VWR (International GmbH, Darmstadt, Ger-
many). JA, SA, ABA, IAA, ICA, traumatic acid, 1, 1’-carbo-
ylulimidazole, 3-(hydroxymethyl)-pyridazine and fatty
acids were from Sigma (Taufkirchen, Germany). OPDA
was from Cayman Chemicals (Ann Arbor, MI). Cinnamic
acid was from Merck KGaA (Darmstadt, Germany). Gib-
berellins was from Carl Roth GmbH (Karlsruhe, Ger-
many). Traumatin was from Larodan Chemicals (Malmö,
Sweden). Traumatol was synthesized by the reduction of
traumatin with NaBH₄ using standard conditions and
²H₂-OPDA 8:0 was synthesized by deuteriation of OPDA
using standard conditions.

Leaf extraction
Extractions were performed according to [13] with the
modifications adopted by [14]. Depending on the experi-
ment, different amounts of frozen leaf material were
homogenized in 2 mL microcentrifuge tubes (Eppendorf,
Hamburg, Germany) containing 2 steel beads (ASK, Korn-
tal-Muenchingen, Germany) by grinding in a Geno-
rinder (SPEX Certi Prep, Metuchen, NJ) for 30 sec at 1300
strokes min⁻¹. After homogenization, samples were
extracted with 1 ml of 10/10/1/1 (v/v/v/v) chloroform/
methanol/acetic acid/water spiked with internal standards
(0.5 μg heptadecanoic acid, 0.4 μg [²H₃]-JA, [²H₃]-SA,
[¹³C₆]-JA-Ile, [²H₄]-ABA, royal jelly acid). After vortexing
for 10 min, the phases were separated by centrifugation at
4 °C for 10 min. The organic phase was transferred into a
fresh tube and the leaf material re-extracted with 1 ml 5/
5/1 (v/v/v) chloroform/methanol/water. After centrifuga-
tion, the organic phases were combined, evaporated to
dryness under a gentle stream of nitrogen and reconsti-
tuted in 0.2 mL of dry dichloromethane for subsequent
derivatization. Quantification was made based on the
internal standards added and calibration curves.

Picolinyl ester derivatization
Formation of Picolinyl ester derivatives of free carboxylic
acids was performed as described in [10]. Briefly, 0.2 mL
of leaf extract in dichloromethane were mixed with 0.1
mL of freshly prepared 1% (w/v) 1, 1’-carbonylulimidida-
zole/dichloromethane. After 1 min, 0.2 mL of freshly pre-
pared 0.1/1/1 (v/v/v) 3-(hydroxymethyl)-pyridazine/
dichloromethane/triethylamine containing catalytic
amounts of 4-(1-pyrrolidinyl)-pyridine were added and
the mixture heated for 10 min at 37°C. The reaction
was stopped by the addition of 25 μL of acetic acid. Samples
were dried under a stream of nitrogen and 0.5 mL of water were added. Picolinyl ester derivatives were extracted twice with 0.5 mL hexane, the solvent evaporated under a stream of nitrogen and the samples reconstituted in 70/30 (v/v) methanol/water for LC-MS/MS analysis.

**LC-MS/MS analysis**

Picolinyl ester derivatives were analyzed in an LC-MS/MS system (Varian 1200 Triple-Quadrupole-LC-MS system; Varian, Palo Alto, CA, USA [http://www.varianinc.com]). Ten μL of the sample were injected onto a ProntoSIL® column (C18; 5 μm, 50 × 2 mm, Bischoff, Germany; [http://www.bischoff-chrom.de]) attached to a precolumn (C18, 4 × 2 mm, Phenomenex, USA). As mobile phases 0.05%/1% (v/v/v) formic acid/methanol/water (solvent A) and methanol (solvent B) were used in a gradient mode with the following conditions: time/concentration (min/%) for B: 0.0/15; 1.5/15; 4.5/98; 19.5/98; 20.5/15; time/flow (min/mL): 0.0/0.4; 1.0/0.4; 1.5/0.2; 18.5/0.2; 19.5/0.4; 25/0.4. To minimize contaminations the solvent eluting from the column was injected into the mass spectrometer only between 1.5 and 18.5 min. Between 0 and 1.5 min and 18.5 and 25 min a mixture of 1/1 (v/v) methanol/water was injected to flush the MS/MS system.

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**Figure 4**

Example of the linear correlation between the amounts of leaf tissue extracted from FAC elicited *N. attenuata* leaves and the amounts of compounds detected. Different amounts of leaf tissue (5 to 250 mg; fresh weight) from *N. attenuata* plants were collected after 60 min of FAC elicitation. Leaf material was extracted, derivatized and analyzed by LC-MS/MS. Two biological replicates were performed for each amount of tissue.
The MS was used in ion positive mode and ions detected using multiple reaction monitoring (MRM) and their respective m/z transitions [M+H]+ > 92 (Table 1) after collision induced fragmentation with argon gas under -25.5 V collision energy. For ionization, the needle was set at 5000 V and the drying gas (nitrogen) at 300 °C and 20 psi (housing 50 °C). The detector was set at 1800 V. Samples were kept at room temperature (RT) and separation was also performed at RT. Analysis of underivatized phytohormones from crude leaf extracts was performed as previously described [6].

**Analysis of FFA by GC-MS**

FFA extraction was performed according to [14]. Heptadecanoic acid (0.5 μg per sample) was added as internal standard for quantification. After extraction, samples were reconstituted in 0.2 mL of chloroform and lipids were separated on Partisil® K6 silica plates (Whatman, Dassel, Germany) which were developed with 70/30/1 (v/v/v) hexane/diethyl ether/acetonic acid. Commercial FFAs were used as standards and plates were stained with 2% (v/v) 2’,7’-dichlorofluorescin/methanol and lipids were visualized under UV light. FFA were eluted from the silica with 3 mL of 2/1 (v/v) chloroform/methanol and methylated in 1% (v/v) H2SO4/methanol for 1 h at 75°C. Fatty acid methyl esters were extracted with hexane and analyzed in a Varian CP-3800 GC coupled with a Varian Saturn 3800 ion trap MS in electron ionisation (EI; 70 eV) mode (Varian, Palo Alto, CA). One μL of the sample was injected in splitless mode on a DB-WAX column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Agilent, Boeblingen, Germany) with helium at a constant flow of 1 mL min⁻¹ as the carrier gas. The injector was at 230°C. The oven temperature program was: 130°C for 5 min, 220°C at 3.0°C/min, 5°C/min ramp to 240°C and hold for 1 min. EI spectra were recorded on Scan mode from 40 to 400 amu. Quantification was performed in the linear range of detection and based on calibration curves generated with increasing concentrations of commercial FAMES mixes (Matreya, Pleasant Gap, PA) and the IS (17:0).

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

MK carried out the experiments and drafted the manuscript. ITB participated in the design and coordination of the study and helped to draft the manuscript. GB conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

**Additional material**

**Additional file 1**

Table S1: Coefficients of variation (CV) of replicate measurements for standard mixtures at different concentrations and leaf sample. Table S2: Analysis of derivatized N. attenuata leaf extracts after multiple extractions and calculation of recovery rates. Table S3: Analysis of different amounts of N. attenuata leaf material.

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**Acknowledgements**

The Max Planck Society is acknowledged for funding.

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