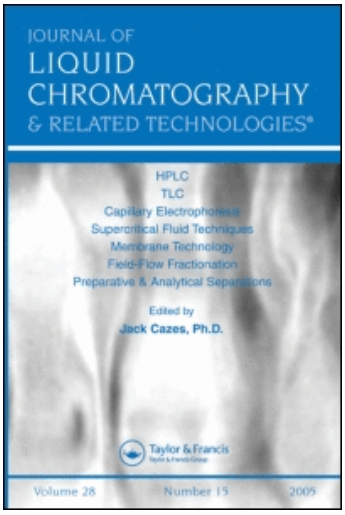

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Vincentius A. Halim[a]; Marianne C. Verberne[a]; Robert Verpoorte[a]
[a] Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, RA, The Netherland

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Differential Metabolic Profiling by HPLC–PDA–MS of Wild Type and Transgenic Tobacco Plants Constitutively Producing Salicylic Acid

Vincentius A. Halim, Marianne C. Verberne, and Robert Verpoorte*

Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, The Netherlands

ABSTRACT

The metabolite profiles of wild type tobacco plants and transgenic tobacco plants, constitutively producing salicylic acid (SA), were compared by means of liquid chromatography with online photo diode array and mass spectrometer detector (LC–PDA–MS). Statistical analysis of the peak area of 15 major peaks showed significant differences in quantities for three compounds, which are present in the extract before acid hydrolysis, containing mainly aglucones. By comparison of the retention time, UV spectra, and molecular weight with reference compounds, two of these

*Correspondence: Robert Verpoorte, Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Leiden University, Einsteinweg 55, P. O. Box 9502, 2300 RA, Leiden, The Netherland; E-mail: verpoort@lacdr.leidenuniv.nl.

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peaks were identified as chlorogenic acid and rutin. The extracts obtained after acidic hydrolysis did not show any significant difference.

Key Words: Metabolic profiling; Tobacco; Transgenic plants; Salicylic acid overexpression.

INTRODUCTION

Genetic engineering of plant secondary metabolism has shown to be a feasible approach for altering secondary metabolite production (for reviews see [1–3]). Generally, in such experiments, products of the pathway of interest are analyzed in the transgenic plants. But, in transgenic plants, many other pathways might also be affected directly or indirectly. To learn more about the overall regulation of the metabolism, and in case of plants eventually aimed for use for human consumption, it will be very useful to map all possible changes in levels of the compounds present. Metabolic profiling, thus, has become an important item for studies of transgenic plants.[4,5] But, also in connection with functional genomics metabolic profiling, aiming at mapping the total metabolome (all metabolites present under a certain condition) of a plant will be very important, as it is the only unbiased method to define the phenotype of a plant. By comparing the metabolome under different conditions, and relating this to the transcriptome (all mRNAs present under these conditions), links can be made between genes and the metabolic pathway they are involved with.

To measure the metabolome, various approaches can be used. But, so far there is no single method that can deal with the challenge of analyzing all metabolites present in a plant, which might be as many as a plant has genes (ca 30,000). Different polarity, large range of concentrations, and instability of the compounds are just some of the problems involved. To overcome these problems, many different approaches can be thought of. These can be divided into two groups, targeted and non-targeted profiling. In the first, methods are selected aimed at the analysis of specific groups of compounds and by using reference compounds, quantitation can be made. In the non-targeted approach, one uses a broader, more general method in which a whole range of compounds can be detected. By means of their physical data (e.g., mass- or UV-spectra) compounds are identified. In this case, one can also decide only to identify those compounds that are different in the different phenotypes analyzed. Such a differential display method matches well with the common practice in transcriptomics and proteomics.

Here, we will describe such a differential metabolic profiling approach to identify difference between wild type tobacco plants, and tobacco plants

(CSA tobacco) that overexpress microbial genes that encode isochorismate synthase and isochorismate pyruvate lyase from microorganisms.[6] These plants constitutively produce high concentrations of salicylic acid (SA) and its glucoside. In plants, SA acts as a signal compound.[7] In tobacco after infection with the tobacco mosaic virus, SA is produced, which makes other parts of the plant become more resistant. Also, the CSA tobacco plants are more resistant against viral and fungal infections.[6] As such defense may also include secondary metabolites, we were interested to see, if by a non-targeted approach, differences in the metabolic profiles could be observed. By a targeted approach,[8–10] only for flavonoids and chlorogenic acid could a difference be found. For the non-targeted approach, HPLC with a gradient elution system in combination with photodiode array and mass spectrometric detection (HPLC–PDA–MS) was used to analyze extracts, using solvents that would favor the extraction of compounds of intermediate polarity. As often such compounds occur both as aglucone and as glycoside, the extracts were also subject to hydrolysis, to identify possible glucosides.

EXPERIMENTAL

Tobacco Leaf Material

Wild type tobacco plants (*Nicotiana tabacum* cv. Samsun NN) and two constitutively producing salicylic acid (CSA) tobacco lines (line 16, F1-generation; line 16, F2-generation; line 14, F2-generation) were germinated on agar medium. The CSA-plants were selected for kanamycin resistance. The seedlings were kept on agar medium for three weeks before they were moved to the soil. The plants were grown under 16 hours light per day (5000 to 6000 lux), the humidity was maintained at 65%. The temperature was 23°C during the light period and 21°C during the night.

After eight weeks of growth in soil, the plants were sampled. Every line was represented by three plants, and from every plant the lower leaves (10th leaf from the bottom, the bottom third part of the plant) and the upper leaves (3rd leaf from the top) were harvested and stored at −80°C.

Chemicals

Ethyl acetate, cyclohexane, acetic acid, and hydrochloric acid were purchased from J. T. Baker (Deventer, Holland), trichloroacetic acid (TCA)

from Merck (Darmstadt, Germany), and HPLC grade methanol from Rathburn (Walkeburn, Scotland). Salicylic acid, vanillic acid, 2,3-dihydroxy benzoic acid, 2,5-dihydroxy benzoic acid, phenyl pyruvate, sinapinic acid, phenylalanine, *para*-hydroxy benzoic acid, *ortho*-coumaric acid, phenyl acetic acid, caffeic acid, and ferrulic acid were from Sigma (St. Louis, MO, USA). Chorismic acid, benzoic acid, nicotine, catechol, and coniferyl alcohol came from Sigma-Aldrich (Steinheim, Germany), anabasine from Aldrich (Milwaukee), and tyrosine from Merck (Darmstadt, Germany). *para*-Coumaric acid, protochatecuic acid, and anthranilic acid were obtained from Fluka (Buchs, Germany). Rutin was purchased from ICN (Ohio, USA) and *para*-amino benzoic acid from Janssen (Beerse, Belgium). Chlorogenic acid was isolated in our laboratory and identified by means of its spectral data.

Salicylic Acid Extraction and Detection

Salicylic acid was extracted according to the method described by Verberne et al.[11,12] The frozen leaf material was pulverized in liquid nitrogen with a pestle and mortar. Samples of 0.5 g were further homogenized and transferred to a 1.5 mL Eppendorf tube. One milliliter of 90% methanol and 2.5 μL of the internal standard 2,3-dihydroxybenzoic acid (10 μmg/μL) were added. The tube was vortexed for 1 min and sonicated for a further 5 min. The tubes were centrifuged for 5 min at maximum speed in an Eppendorf table top centrifuge. The supernatant was collected in a 2 mL Eppendorf tube. The pellet was resuspended in 0.5 mL 100% methanol, and again treated in the same way. The two supernatants were combined and taken to dryness in a SpeedVac concentrator. The residue was vortexed with 250 μL 5% trichloroacetic acid in water, and 800 μL ethyl acetate–cyclohexane (1 : 1) was added. The organic layer was removed and the partitioning was repeated once more. The organic layers were combined and taken to dryness. The aqueous layers were combined and hydrolyzed by adding 300 μL 8 M HCl and heated at 80°C for 1 h. The hydrolyzed fraction was extracted as described above. The dried organic fractions were dissolved in 600 μL of the HPLC eluent prior to analysis. The HPLC system consisted of a Phenomenex column type Luna 3u C18(2) 150 × 4.60 mm 3 μm. The eluent contained 60% acidified water (pH 2.8, using acetic acid) and 40% methanol. The flow rate was 0.7 mL/min. Twenty microliters of the extract was injected. Salicylic acid was detected with a Shimadzu RF-10Axl spectrofluorometric detector, using an excitation wavelength of 300 nm and an emission wavelength of 410 nm.

Extraction Method of Free and Bound Compounds

Free- and bound-compound (extract obtained after hydrolysis) extracts were prepared according to the extraction method of SA, as described above. After evaporation of the ethyl acetate-cyclohexane fractions, the samples were dissolved in the HPLC-eluent (5% methanol + 95% acetic acid acidified water (pH 2.8).

HPLC System for Profile Analysis of Tobacco Leaf Extract

Injections of 50 μL were made on a Phenomenex Column type Luna 3 μ C18(2) 150 × 4.60 mm 3 μm. The flow rate was 0.7 mL/min, with a gradient elution from 100% solvent A (5% Methanol + 95% acetic acid-acidified water pH 2.8) to 100% solvent B (99% methanol + 1% acetic acid) (Table 1). The samples were analyzed using a Waters 991 PDA detector (Milford, MA) with a wavelength range of 210 nm to 350 nm.

Mass Spectrometric System

The HPLC system was connected to a MS for LC–MS analysis. A Finnigan MAT (San Jose, CA) TSQ-700 triple quadrapole MS equipped with an electrospray (ESI) ionization interface was used. The ESI values consisted of an electrospray voltage of 4.27 kV with a heated capillary temperature of 200°C. Nitrogen was used as a sheath gas. Full scan spectra from m/z 50 to m/z 800 in the positive ion mode were obtained (scan time 3 s).

Table 1. Stepwise gradient in the HPLC system of 100% solvent A (5% methanol + 95% acetic acid—acidified water pH 2.8) to 100% solvent B (99% methanol + 1% acetic acid).

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
5	100	0
30	65	35
40	65	35
50	0	100
60	0	100

Statistical Analysis

The HPLC data of the lower and upper leaves, and of free and bound compounds at 280 nm were used to perform a statistical analysis of the HPLC-profiles. Multiple comparison-ANOVA and a LSD (Fisher) test with $\alpha = 0.05$ were used to compare the average peak areas ($n = 3$) of the corresponding peaks between wild type and CSA line 16-F1, CSA 16-F2, and CSA 14-F2 tobacco plants.

RESULTS AND DISCUSSION

For the extraction, a method was chosen in which compounds with an intermediate polarity would be obtained. To avoid problems with chlorophyll and sugars, major compounds in the leaves, the methanolic extract was taken to dryness and subsequently partitioned between water and an organic solvent. To also be able to detect the aglycones of possible glycosides that remained in the water layer, this water layer was hydrolyzed and again extracted with organic solvent.

The free- and glucosidically-bound SA content in wild type and CSA tobacco line 16 (Fig. 1) was determined to investigate whether the CSA plants used in this study produce salicylic acid.[6,11,12] The high content of salicylic acid in CSA line 16-F1 and in line 16-F2, compared to the salicylic acid levels in wild type tobacco plants, proves that the inserted genes are still active in the transgenic plants. The large standard deviation of SA in CSA line 16-F2 is caused by the large variation among different F2-plants. CSA line 14-F2 also accumulated SA (data not shown). The leaf material of the three CSA lines was used to investigate the influence of constitutive SA production on the metabolic profile of tobacco.

For the metabolic profiling of the leaves, an HPLC gradient system was developed by which a variety of compounds in the tobacco leaf extracts could be separated (free- and bound-compounds extract). A series of known standards were analyzed in this system (Table 2). This gradient-HPLC system contains only water, methanol, and acetic acid, which make it possible to couple the HPLC system with MS.

The reproducibility of the developed HPLC-method was examined by analyzing the percentage of variability in peak area of a peak in the free-compounds extract. This peak was located in the middle of the chromatogram and had a retention time of 28.06 ± 0.11 min. Four identical extracts were analyzed intraday and seven identical extracts were analyzed interday. A variability of 9.2% was found both for intraday and interday analysis.

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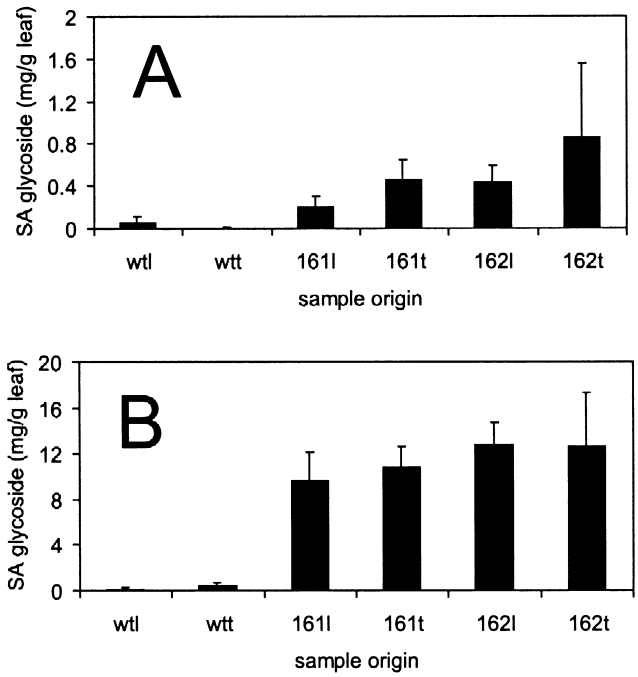

Figure 1. Free SA content (A) and SA released after acid hydrolysis (B) in wild type and CSA tobacco plants of line 16-F1 and line 16-F2. l = lower leaf, t = upper leaf.

The reproducibility of the peak area and the consistency of the peak pattern were good, so we concluded that this gradient HPLC system was suitable for profiling the tobacco plants.

For the profiling, all samples (free and bound-compounds extracts of upper and lower leaves of wild type and CSA type tobacco) were analyzed using this gradient HPLC system coupled with PDA detection. For all major peaks, the UV spectra were recorded. Based on this, a wavelength of 280 nm was selected for quantitation, at which aromatic compounds usually have UV absorption. Comparing the chromatograms, a number of peaks could be observed, which have different peak areas in the wild type and CSA plant extracts. From the free-compounds extract (Fig. 2), 15 unknown peaks were selected for further analysis. From the bound-compounds extract (Fig. 3) 25 unknown peaks were selected for further analysis.

In Figs. 4 and 5, the differences in peak area of the selected peaks in the metabolic profile of the lower leaf extracts between wild type and CSA line

Table 2. Compounds that can be analyzed by the HPLC system, using the gradient as shown in Table 1.

	Reference compounds	Molecular weight	Retention time (min)
1	*p*-Amino benzoic acid	137	9.09
2	Protochatecuic acid	154	16.49
3	Cathecol	110	18.22
4	Tyrosin	181	20.00
5	*p*-Hydroxy benzoic acid	138	24.72
6	2,5-Dihydroxy benzoic acid	154	25.51
7	Chorismic acid	226	25.69
8	Chlorogenic acid	354	28.49
9	Anthranilic acid	137	30.08
10	Vanillic acid	168	29.43
11	Caffeic acid	180	31.00
12	2,3-Dihydroxy benzoic acid	154	31.09
13	Phenyl pyruvic acid	164	33.00
14	Coniferyl alcohol	180	34.30
15	Synapinic acid	224	35.96
16	*p*-Coumaric acid	164	37.05
17	Ferrulic acid	194	38.51
18	Phenyl acetic	136	40.98
19	Benzoic acid	122	42.00
20	Rutin	610	45.50
21	Salicylic acid	138	46.00
22	*o*-Coumaric acid	164	46.77
23	*t*-Cinnamic acid	148	52.00

16-F1 are presented. To perform an unbiased analysis, a multiple comparison-ANOVA and LSD (Fisher) test with $\alpha = 0.05$ was performed to compare the average peak areas of compounds in the extracts of the lower leaf and upper leaf of wild type and CSA (line-16-F1) tobacco plants. After statistical analysis of the data, three significantly different peaks were found in the free-compounds extracts (peak number 6, 8, and 12), both in lower and upper leaf (Fig. 6). Peak number 11 is only significantly different in the upper leaves. Also, CSA 16-F2 and CSA 14-F2 show lower levels in top leaves, compared to lower leaves and lower levels in CSA lines compared to the wild type levels.

Different from the free-compounds extracts, the extracts (bound-compounds extract) obtained after acid hydrolysis showed a smaller difference. The only significantly difference after acid hydrolysis is peak number 4

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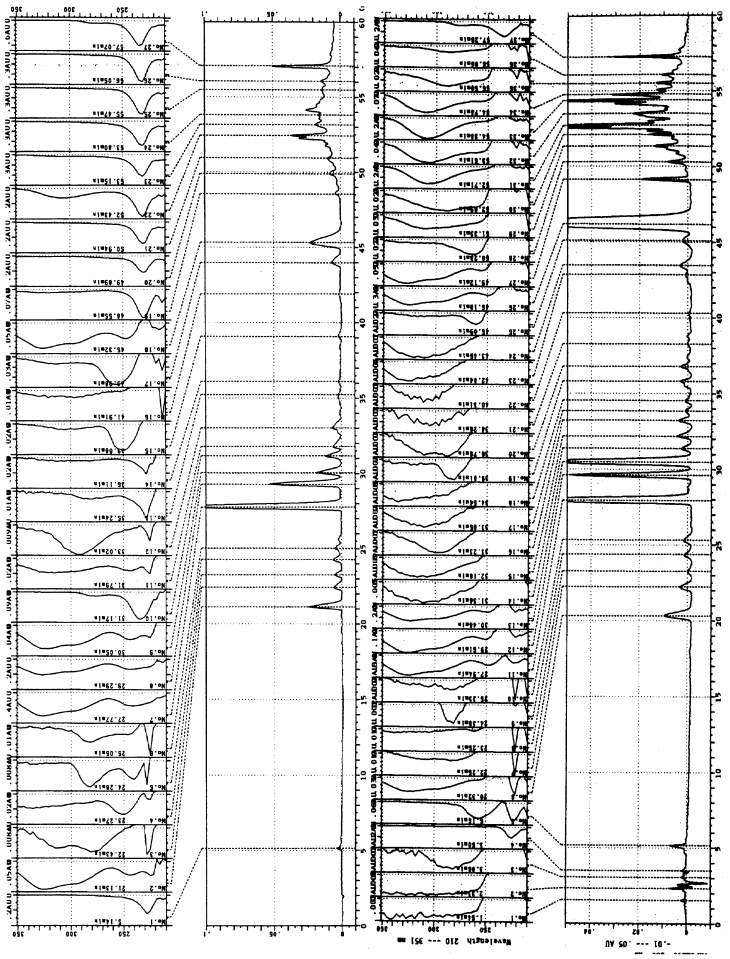

Figure 2. HPLC-chromatogram of the free compounds extract from the lower leaves of a CSA tobacco plant, line 16-F1 (upper part), and of a wild type tobacco plant (lower part).

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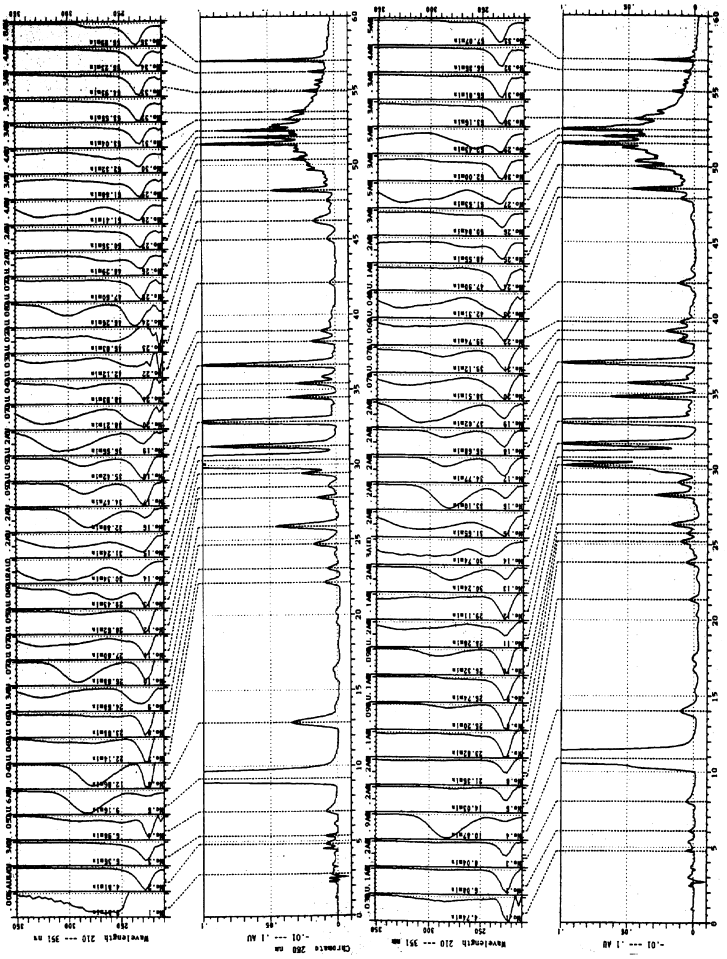

Figure 3. HPLC-chromatogram of the bound compounds extract from the lower leaves after acid hydrolysis of a CSA tobacco plant, line 16-F1 (upper part), and of a wild type tobacco plant (lower part).

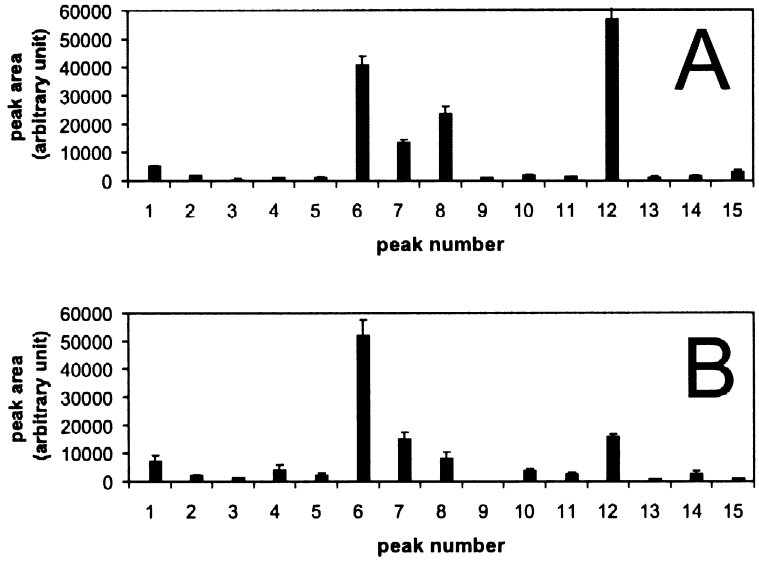

Figure 4. Average peak area of 15 peaks selected from the HPLC-chromatogram of the free compounds extract of lower leaves, both in wild type tobacco plants (A) and in CSA tobacco plants, line 16-F1 (B) ($n = 3$).

in the upper leaf extracts. Based on this, we can conclude that the free compounds in CSA plant line 16-F1 are influenced more by the production of SA than the bound compounds.

The next step after profile analysis was the identification of the peaks that were different in both upper and lower leaves (peaks and 12). Based on the UV-spectra, they were thought to be phenolic compounds. After comparison of the retention time, UV spectra and molecular weights (Table 3) between the peaks of interest and the reference compounds, two peaks in the free form compound extract were identified as chlorogenic acid (peak 6) and rutin (peak 12). Spiking with reference compounds further confirmed the identification.

The third peak could not be identified since there is no reference compound in our library, which matches with this peak. The UV spectra of this peak was similar with the UV spectra of chlorogenic acid, this compound might, thus, be a derivative of chlorogenic acid.

The lower concentrations of chlorogenic acid and rutin in the transgenic tobacco plants, compared to wild type tobacco plants might be caused by the channeling of chorismate towards SA. However, the levels of SA are rather low compared to the known capacity of the phenylpropanoid pathway, that can

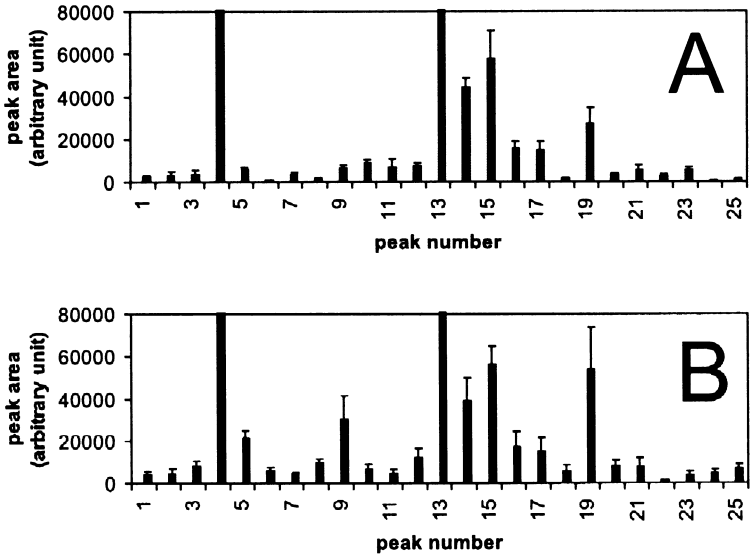

Figure 5. Average peak area of 25 peaks selected from the HPLC-chromatogram of the bound compounds extract of lower leaves both in wild type tobacco plants (A) and in CSA tobacco plants, line 16-F1 (B) ($n = 3$).

channel as much as 20% of the total carbon flux of the plant cell towards various phenolic compounds. With the large differences in the flux between the isochorismate and the prephenate pathways, an effect of SA as signal compound on the biosynthesis of chlorogenic acid and rutin seems a more likely explanation.

CONCLUSION

Using online LC–PDA–MS and a differential analysis of the peak areas, we observed different profiles for wild type tobacco plants and the CSA tobacco plants. The compounds, which differed between the two types of plants, were identified as chlorogenic acid and rutin. The results of the differential metabolic profiling has led us to the same results as obtained by a targeted approach.[8–10] Although, this approach gives us an insight in the differences between the two plants, by choosing an extraction method, one makes a choice for a certain polarity window of the compounds extracted. The total metabolome of the plant is much larger than about the 60 peaks observed

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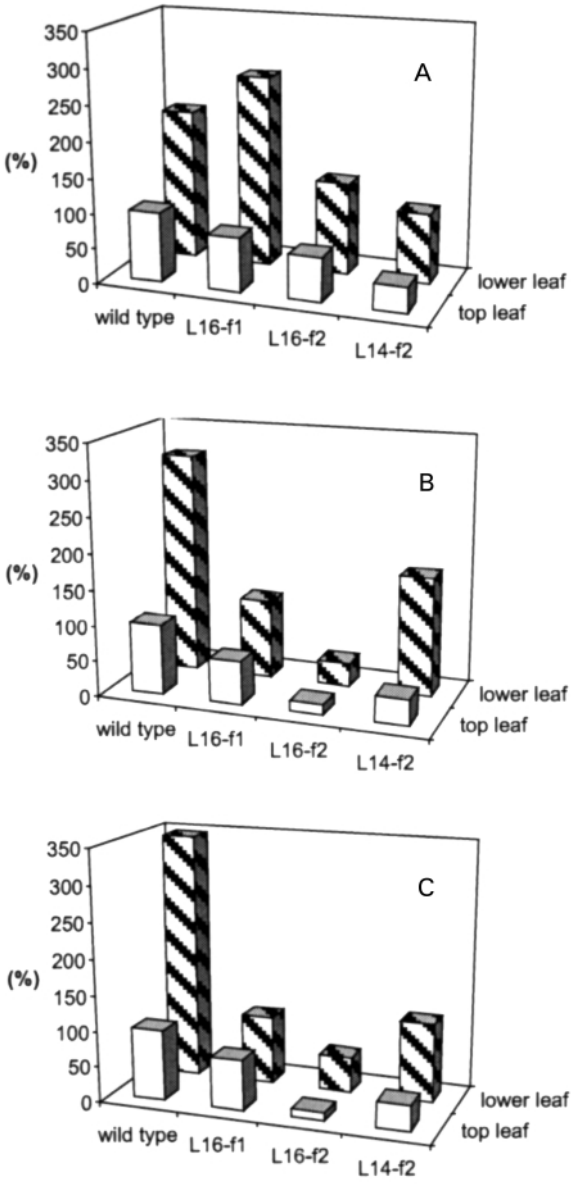

Figure 6. Comparison of the peak area of peak number 6 (A), 8 (B), and 12 (C) from the free compound extracts of wild type tobacco plants, and of CSA tobacco plants of line 16-F1, 16-F2, and line 14-F2.

Table 3. Online LC–PDA–MS analysis of free-compounds extract from tobacco leaves.

Peak	Retention time (min)	Std. Dev. (min)	Molecular weight
1	21.13	0.7	246, 260, 332, 346, 389, 476, 488, 561, 617, 688
2	22.67	0.4	
3	23.62	0.32	
4	24.63	0.24	
5	25.61	0.34	
6	28.06	0.11	354, 376, 512, 730,
7	29.67	0.07	411, 425, 497, 510
8	30.54	0.07	204, 222, 244
9	31.38	0.06	
10	32.15	0.06	
11	33.23	0.04	
12	46.04	0.16	610
13	43.06	0.41	
14	44.88	0.2	246, 260, 332, 346, 510, 583
15	48.98	0.12	

in the method here described. A number of complementary methods will be necessary to deal with the whole range of compounds with different polarities.

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